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TESIS DOCTORAL

Identificación y caracterización de fuentes de resistencia a estreses bióticos y abióticos en guisante

Identification and characterization of resistance to biotic and abiotic stresses in pea

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TITULO: *Identification and characterization of resistance to biotic and abiotic stresses in pea*

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TÍTULO DE LA TESIS:
Identificación y caracterización de fuentes de resistencia a estreses bióticos y abióticos en guisante

DOCTORANDO/A: Thaïs Aznar Fernández

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

El trabajo titulado "Identificación y caracterización de fuentes de resistencia a estreses bióticos y abióticos en guisante" se considera finalizado y reúne los requisitos para su exposición y defensa como tesis Doctoral.

Esta tesis ha obtenido resultados relevantes para la mejora de guisante para resistencia a plagas. Se han identificado nuevas fuentes de resistencia a gorgojo y pulgón y se han caracterizado los mecanismos de resistencia implicados en dicha resistencia. Estos trabajos han requerido el aprendizaje de distintas técnicas tales como la evaluación de germoplasma en campo y condiciones controladas, el desarrollo y análisis de marcadores moleculares y el manejo de distintos programas y análisis estadísticos. Los resultados de la tesis han dado lugar ya a dos artículos en prensa en revistas SCI del primer decil, y otras tres en fase de evaluación o envío. Además, los resultados se han presentado en varios congresos internacionales. Dichas contribuciones se reseñan a continuación:

1. Aznar-Fernández et al., 2017. Identification and multi-environment validation of resistance to pea weevil (*Bruchus pisorum*) in *Pisum* germplasm. **Journal of Pest Science**, on line first. https://doi.org/10.1007/s10340-017-0925-1, *IF_{2016}=3.73, Ranking 4/91 (Entomology),* ***D1***
2. Aznar-Fernández & Rubiales, 2018. Identification and characterization of antixenosis and antibiosis to pea aphid (*Acyrthosiphum pisum*) in *Pisum* spp. germplasm. **Annals of Applied Biology**, in press, DOI: 10.1111/aab.12417*; IF_{2016}=2.05, Ranking: 5/57 (Agriculture multidisciplinary),* ***D1.***
3. Aznar-Fernández & Rubiales, … Influence of flower and pod source on pea weevil (*Bruchus pisorum*) oviposition capacity and preference. En evaluación en Annals of Applied Biology.
4. Aznar-Fernández et al., … Antifeedant activity of long-chain alcohols, and fungal and plant metabolites against pea aphid (*Acyrthosiphon pisum*) as potential biocontrol strategy. En evaluación en Natural Products Research.
5. Aznar-Fernández et al., … Identification of quantitative trait loci (QTL) controlling Seed infestation and larval development of pea weevil (*Bruchus pisorum*) in a high-density integrated DArTseq SNP-based genetic map of pea. En fase final de redacción

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 18 de Febrero de 2018

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TÍTULO DE LA TESIS:
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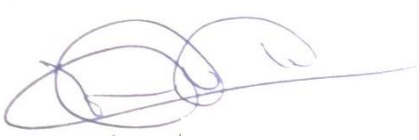

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Abstract

Pea (*Pisum sativum* L.) is the most widely cultivated temperate grain legume in Europe and the second in the world, it is used for animal feed and human food. As a legume, it is a source of protein and it brings environmental benefits being a safe bet for sustainable agriculture. However pea production is severely affected worldwide by abiotic and biotic stresses. The goal of the thesis is the identification and characterization of resistance to two major insect pests: *Bruchus pisorum* (pea weevil, *Bp*) and *Acyrtosiphon pisum* (pea aphid, PA), that cause yield and quality losses worldwide.

Bp is a specialized pest which larvae feed into seeds decreasing the quality and marketability of the seeds and may cause losses of up to 50% of the harvest. Moreover a constant monitoring and treatment in the field and also in harvest period are needed. On the other hand PA is a phloem sucking pest that causes nutrient deficiencies and plant stunting which causes significant yield losses and transmit viruses. Being a polyphagous pest with a huge environmental adaptation, multiple treatments are necessary in the field.

The little performance gained with biological control and the lack of resistance in elite cultivars prompted to look for resistance in wild genotypes and landraces. For this reason, this thesis has the objective of expanding the knowledge of plant-insect interactions as well as obtaining new sources of resistance to implement them in breeding programs, with the long term goal of develop resistant varieties and reduce the economic and ecologic costs which supposes its control. Following these

objectives, a collection of *Pisum* spp. was screened in different plots assays to identify and characterize pea resistance against both pests.

Chapter 1 deals with screening for *Bp* resistance in a multi-environment field tests. Assessments on seed infestation (SI) and larval development (LD) were subjected to a heritability-adjusted genotype and genotype x environment biplot analysis. Results showed that both traits were independent and were influenced by environmental traits. An accurate canonical correspondence analysis showed that both decreased in rainy seasons, meanwhile accumulated radiation and photoperiod favoured SI and decreased LD. Some accessions from different *Pisum sativum* subspecies highlighted with stable low values SI and / or LD.

Chapter 2 describes choice and no choice bioassays to discern possible effects of flower and pod genotype on oviposition. Assays were performed with resistant accessions selected from chapter 1 under controlled conditions. No choice assays showed a significant genotypic effect on oviposition for both, being reduced to half on pods of particular pea accessions and negligible on pods on other legumes (*Lathyrus sativus* and *Vicia faba*). Dual choice assays also showed significant differences in oviposition preference. Data form chapter 2 could be useful for crops distribution and has increased the knowledge between *Bp* and pea.

Chapter 3 describes the identification of Quantitate Trait Loci (QTL) for *Bp* resistance by phenotyping SI and LD in a Recombinant Inbred Line population (RIL) already genotyped using EST's, SSR and DArT markers. Results elucidated three linkage groups (LG) involved in *Bp* infestation and one LG involved in larval development.

These markers provide a highly valuable resource for future smart breeding approaches against *Bp*.

Chapter 4 deals with identification of resistance to PA in *Pisum* spp. collection. Field screenings were performed over two seasons at Córdoba. The most resistant accessions were further characterized under semi-controlled conditions for PA coverage, reproduction and plant damage. Results elucidated the valuable resistance of accessions P40 and P665, with the combination of both antixenosis, by reducing aphid preference, and antibiosis, by diminishing aphid proliferation.

Chapter 5 deals with the study of feeding repellence and toxicity to PA of several long chain alcohols (LCOH) and metabolites (derived from different organisms) chosen on the basis of their previous biological activity described. Results elucidate high feeding deterrence by some of the compounds. On the contrary, aphid mortality was low although significant for some of them. In addition phytotoxic effect on plants was evaluated elucidating Seridin as the best candidate.

Resumen

El guisante (*Pisum sativum* L.) es una de las leguminosas de grano de clima templado más cultivada en Europa y la segunda en el mundo. Su uso se extiende al consumo humano y animal. Además al ser una legumbre aporta elevadas cantidades de proteína, así como beneficios medioambientales convirtiéndola en una apuesta segura para una agricultura más sostenible. No obstante el cultivo de guisante se ve severamente afectado a nivel global por un conjunto de estreses bióticos y abióticos. Esta tesis se centra en dos de las plagas que más merman la calidad y el rendimiento de este cultivo: *Bruchus pisorum* conocido como el gorgojo del guisante y *Acyrtosiphon pisum,* popularmente nombrado como pulgón verde del guisante.

El gorgojo es un insecto monófago cuya larva se alimenta del cotiledón de las semillas, dejándolas huecas, reduciendo así la calidad y valor de mercado de las mismas, pudiendo causar pérdidas superiores al 50% en los cultivos. Además, requiere de numerosos tratamientos, en campo y en post cosecha, para controlarla. Por otro lado el pulgón verde del guisante es un insecto que se alimenta de la savia de las plantas mediante un aparato bucal succionador, causando deficiencias nutritivas que debilitan a la planta, lo que conlleva grandes pérdidas de producción, además de ser transmisores de virus. El pulgón se caracteriza por ser polífago con una capacidad extraordinaria para adaptarse al ambiente, todo ello hace que para combatir esta plaga sean necesarios múltiples tratamientos en campo.

El bajo rendimiento que se obtiene con el control biológico junto con la falta de resistencias en los cultivares han promovido la búsqueda de resistencias en genotipos silvestres y variedades población. Por este motivo, el objetivo de esta tesis es ampliar

el conocimiento sobre la interacción planta-insecto, así como identificar nuevas fuentes de resistencia para incorporarlas en programas de mejora con el objetivo a largo plazo de desarrollar variedades resistentes y reducir los elevados costes ecológicos y económicos que supone el control de ambas plagas. Para ello se ha evaluado una colección de *Pisum* spp. en distintos ensayos para identificar y caracterizar nuevas resistencias en guisantes contra estas dos plagas.

El **capítulo 1** se basa en la búsqueda de resistencias contra el gorgojo bajo condiciones de campo en un análisis multi-ambiente. Las evaluaciones de nivel de infestación (SI, del inglés Seed Infestation) y desarrollo larval (LD, del inglés Larval Development) fueron sometidas a un análisis estadístico biplot de heredabilidad ajustada por genotipo y genotipo x ambiente. Los resultados mostraron que ambos caracteres son independientes y están muy influenciados por el ambiente. Además un estudio de correspondencia canónica aclaró que ambos caracteres disminuyen en estaciones lluviosas, mientras que las estaciones con una elevada acumulación de radiación y fotoperiodo favorecen los niveles de infestación pero disminuyen el nivel de LD. Finalmente ciertas subespecies de *Pisum sativum* mostraron niveles estables de baja infestación y/o desarrollo larval.

En el **capítulo 2** se describen bioensayos de preferencia y no-preferencia para discernir el efecto del genotipo de la flor y la vaina sobre la ovoposición del gorgojo. Los ensayos fueron desarrollados con los genotipos resistentes identificados en el capítulo 1 bajo condiciones controladas. Los ensayos de no preferencia mostraron un efecto significativo en la puesta tanto para el genotipo de la flor como para el de la vaina, siendo reducida a la mitad en ciertos genotipos y hasta despreciable en vainas

de otras leguminosas (almorta y haba). Los bioensayos de preferencia también mostraron diferencias significativas en la preferencia de ovoposición. La información de este capítulo puede ser útil a la hora de distribuir distintos tipos de cultivos además de haber incrementado el conocimiento de la interacción gorgojo-guisante.

El **capítulo 3** describe la identificación de QTL (del inglés Quantitate Trait Loci), para gorgojo mediante la caracterización de SI y LD de una población RIL (del inglés Recombinant Inbred Line) que previamente ya había sido genotipada con marcadores moleculares EST's, SSR y DArT. Los resultados mostraron tres grupos de ligamiento (LG, del inglés Linkage Group) implicados en el nivel de infestación del gorgojo (SI) y un LG implicado en el desarrollo larvario (LD). Los marcadores encontrados son de gran valor para desarrollar programas de mejora más dirigidos contra el gorgojo.

El **capítulo 4** consiste en la identificación de resistencia contra pulgón en condiciones de campo en una colección de *Pisum* spp. durante dos años. Posteriormente, los genotipos más resistentes fueron evaluados bajo condiciones semi-controladas y controladas evaluando la reproducción y cobertura de pulgón así como el daño en planta. Los resultados mostraron valiosas resistencias en dos genotipos P40 y P665, combinando antixenosis, mediante la reducción de la preferencia del pulgón, y antibiosis, disminuyendo la reproducción de los mismos.

El **capítulo 5** trata sobre el estudio de varios compuestos naturales y su capacidad disuasoria o tóxica sobre el pulgón verde del guisante. Los compuestos derivados de alcoholes de cadena larga (LCOH) y toxinas (provenientes de hongos o plantas), fueron escogidos basándonos en previas actividades biológicas descritas. Los resultados mostraron elevada capacidad disuasoria por parte de algunos compuestos.

Por el contrario la mortalidad fue muy baja, a pesar de mostrar diferencias significativas entre compuestos. Finalmente, los estudios de fitotoxicidad mostraron al metabolito Seiridin como mejor candidato.

Index

General Introduction

1. GENERALITIES OF THE PEA CROP

Pea (*Pisum* spp. L.) belongs to leguminosae family (*Fabaceae*), the third largest flowering plant family with 800 genera and more than 18,000 species (Lewis et al., 2005).

Pea is an important cool season legume crop produced worldwide, mainly in temperate regions (Rubiales et al., 2012). Nonetheless, pea shows a broad distribution, bearing temperatures 7ºC to 30ºC (Slinkard et al., 1994). The cultivated pea is herbaceous specie usually with compound leaves and a variable number of leaflets (1-7) with ending tendrils. However, there is a transformation of this typical model called leafless (Caminero et al., 1997).

As is typical of legume crops, pea is an important source of proteins (20%), carbohydrates (52.7%), fibre (11.3%), minerals (such as iron, zinc, folate, and magnesium) and vitamins (Mudryj et al., 2014, Vaz Patto et al., 2014). In addition, recent novel advances have elucidated the legume contribution in human health by possessing anticancer (Dixon and Sumner, 2003) several bioactive peptides (Malaguti et al., 2014), and resistant proteins involved in immunological and hormonal system or acting as naturally-occurring chemotherapeutic or radioprotective (Clemente et al., 2011). Notwithstanding the above, pea showed anti-nutritive and allergenic compounds (Vaz Patto et al., 2014) such as tannins and phytohemagglutinins that are reduced in quantity by heat treatments, generating tolerable levels for human and animal consumption (Ramos Monreal et al., 1996).

In addition, being a legume, pea brings agro-ecological services linked with its ability to develop symbiotic nitrogen fixation by converting the inert atmospheric nitrogen (N_2) to ammonia (NH_3), making it useful to living organisms and reducing the use of nitrogen (N) fertilizers (Yang et al., 2017). Pea also lays a roll by securing carbon sequestration into food, feed and biofuel crops (Nemecek et al., 2008), as well as plays a critical role in crop rotation (Nemecek et al., 2015). That makes pea a safe bet for a more sustainable agriculture (Rubiales and Mikic, 2014).

Pisum taxonomic classification, has changed over time. The current accepted version comprises three main species, *P. abyssinicum* A. Br. (Maxted and Ambrose, 2001), *P. fulvum* Sm. and *P. sativum* L. The last one can be divided into several

subspecies: *Ps*. ssp. *sativum*, *Ps*. ssp. *elatius*, *Ps*. ssp. *humile*, *Ps*. ssp. *arvense*, *Ps*. ssp. *transcaucasicum* and *Ps*. ssp. *hortense* (Redden et al., 2005, Ambrose, 2008, Rubiales et al., 2012).

1.1. Origin and Domestication

The origin of genetic diversity of pea covers the broad area of the Fertile Crescent through Turkey, Syria, Iraq, Israel and Lebanon. It extends further east to Central Asia (Iran, Afghanistan, Pakistan and Turkmenistan) (Smýkal et al., 2011). A secondary centre of pea diversity is considered in central highland region of Ethiopia and uplands of Southern Yemen, which covers the currently known distributional range of *P. abyssinicum* (Redden et al., 2005, Ambrose, 2008). *P. sativum* ssp. *elatius* and ssp. *sativum* are found naturally in Europe, north western Asia and extend south to temperate Africa. The origin from *P. fulvum* species covers Iran and Turkmenistan through Occidental Asia, north Africa and southern of Europe (Maxted and Ambrose, 2001, Maxted et al., 2010).

Archaeological and genetic research indicates that pea was one of the most significant crops in the human civilization (Mikić et al., 2014). Remainders of this pulse have been found belonging to the tenth and ninth millennium BC, suggesting a previous domestication than for cereals (Zohary et al., 2012). The earliest evidence of peas cultivation is dated 7000-6000 BC, from Neolithic farming villages in northern Iraq, Turkey and Syria (Ambrose, 2008), as well as in Southern Europe (Zohary and Hopf, 1973).

Studies of the domestication of pea by wild pea harvesting (Abbo, et al., 2008, 2011, 2013) suggested the no processed of seeds during the domestication. People nimbly selected for peas that had a softer shell and ripened during the wet season, as wild peas have a hard, water-impermeable seed coat which produce low germination rates (Abbo et al., 2011). Traits such as resistant pods in order to avoid seed scattering, larger production shoot basal branching and reduction of seed toxins and antimetabolites were also selected (Smart, 1990, Zohary and Hopf, 2000, Weeden and Porter, 2007). Nowadays the selection for human consumption is focused on semi-

leafless pea varieties, which are more erect and allow better light and air penetration (Koivisto et al., 2003). Meanwhile for forage production, leafy and taller plants are preferable because a high leaf-to-stem ratio is associated with a higher nutritional value (Collins, 1995).

1.2. Genetics and germplasms

Pisum species are diploid (2n = 14) with a genome size of ca. 4800Mbp (McPhee, 2007), being the majority fully inter-crossable and producing viable hybrids (Redden et al., 2005).

The molecular advances allowed to position *Pisum* between *Vicia* and *Lathyrus* and closely allied to *Vavilovia*. In addition, thanks to the great technological advances, a consistent view of *Pisum* has been reached by developing several DNA markers of pea germplasm. The huge genetic diversity could be structured in degrees of relatedness that reflect taxonomic identifiers, eco-geography and breeding gene pools (De Ron, 2015). A number of genes and marker-trait association have been identified (Tayeh et al., 2015). The recognition of genes involved in specific phenotypes has been propelled by TILLING (Targeting Induced Local Lesions in Genomes). This technique is capable to identify genotypes carrying different alleles of interest by analysing mutations in many individuals for a particular gene, with the long term goal of use them as progenitors in breeding programs (Dalmais et al., 2008).

Due to its genetic diversity (Hancock, 2012), the cultivated *P. sativum* presented a high rank of different morphology with spring and winter varieties, leafy and leafless, early- or late-maturing (Figure 1). As well as for the huge diversity of seeds that can vary in colour, shape and size (see Figure 4 in Chapter 1).

Figure 1. *Pisum* spp. collection sown under field conditions. Pictures show the phenotypic differences 107 days after sown. a: *Ps*. ssp. *elatius* leafy plant, showing late-maturing; b: *Ps*. ssp. *sativum* semi-leaflet plant showing white flowers; c: *P. fulvum* creeping plant of small size with coloured flowers; d: Overview of the experimental field with the pea collection used

In order to preserve the genetic pea diversity, around 28 national and international collections are holding ex situ germplasm consisting on 73,931 pea accessions. However, around 20% of the world's ex situ pea germplasm is duplicative (Smýkal et al., 2013), which imply that there are 59,000 unique accessions known. The four largest active collections include the Australian Temperate Field Crop Collection, Horsham, of 7432 accessions, Vavilov Research Institute of Plant Industry (VIR), St. Petersburg, Russia, with 6790 accessions, the US Department of Agriculture (USDA) 6827 accessions, and the French National Institute for Agricultural Research (INRA) of 8839 accessions held at Dijon, France. In addition there are websites to order readily available *Pisum* accessions. The two sites with highest turnover of international requests are the JI Centre (JIC; http://www.jic.ac.uk/germplasm/) from UK and the USDA (http://www.ars-grin.gov/npgs/), USA.

The John Innes (JI), Norwich *Pisum* collection is very representative and the best studied. It contains 1200 *P. sativum* cultivars, 600 traditional landraces and 750 genetic stocks and reference lines together with wild *Pisum* samples. The International Center for Agricultural Research in the Dry Areas (ICARDA) possesses a large collection of 6105. A very diverse and least duplicative ex situ collection is found in Australia.

2. PEA PRODUCTION AND CONSUMPTION

Pea is an important pulse crop which uses extend to dry peas for animal feed and fodder, forage peas and green peas for human consumption. However, pea varieties can be multi-purpose, such as forage pea that can also be used for green manure (Maxted et al., 2010), fed fresh (grazed or green chop) as hay or ensiled and pea straw (crop residues form the harvest), also known as haulms, that can be fed fresh or ensiled.

The world average of cultivated area for peas reach the 9 millions of hectare, being nearly the 75% of the surface for dry peas with 7.625.705 ha with an average yield of 1.9 tonnes/ha. Green pea acreage is smaller (2.589.087 ha) but average yield much higher (7.68 tonnes/ha) (FAOSTAT 2017) (Figure 2).

Although dry peas are grown commercially in almost 100 countries, its production showed a decreasing trend over the past two decades in Europe. That

could be explained due to different feeding habits jointly with their yield inconsistency and their stood in for soy. Canada, China and Russian Federation have become the world powers for this crop, generating around one half of the world's dry peas (FAOSTAT 2017) (Table 1). On the other hand, green peas have triplicated its cultivated area in the last 50 years (Figure 2), being China the producer of 61% of global green pea production (De Ron et al., 2016). The current dry pea production in Europe is 3 million tons, with Russia standing out with 53% of the total, followed by France (27%) and Ukraine (10%), while 11.4 million are produced in the world tons and the main producing country is Canada (González-Bernal and Rubiales, 2016a).

Table 1. Countries with greater dry pea production in tonnes on 2016 jointly with Europe and Asian region, and the percentage that they represent form the global dry pea production.

DRY PEA PRODUCTION		
	Production (t)	**%**
Canada	4.611.100	32.101
China	1.194.131	8.311
Russian Federation	2.199.489	15.31
U.S.A.	782.388	5.45
Europe	**2.302.098*	**16.02*
Asia	**2.463.400*	**17.15*

*Europe and Asia as a continents facilitate the vision of the huge percentage of production that countries like Canada, China or Russian Federation

In Spain as in Europe, pea cultivation has decreased because of new feeding habits as well as an increment of importations (González-Bernal and Rubiales, 2017) However, the most cultivated legume is still dry pea, which has increased in the last decades, contrasting with the continuous decrease of lentil, faba bean, common bean, lupin (FAOSTAT 2017) (Figure 3 a). The main producers of pea in Spain are Castilla La Mancha and Castilla y León. Severe ups and downs in dry pea cultivation (Figure 3 b) are probably due to the fluctuation of its demand linked to the price of soybeans. Following the laws of supply and demand, when the consumption of soya grows globally, its price also increases (González-Bernal and Rubiales, 2016a). This fact makes

increase the importation and promotes the national production of other legumes, being dry pea the first option due to its potential, adaptation as well as being the most accepted by feed manufacturers, being able to be incorporated up to 30% in pig feed and between 15-20% in those of birds and ruminants (González-Bernal and Rubiales, 2017).

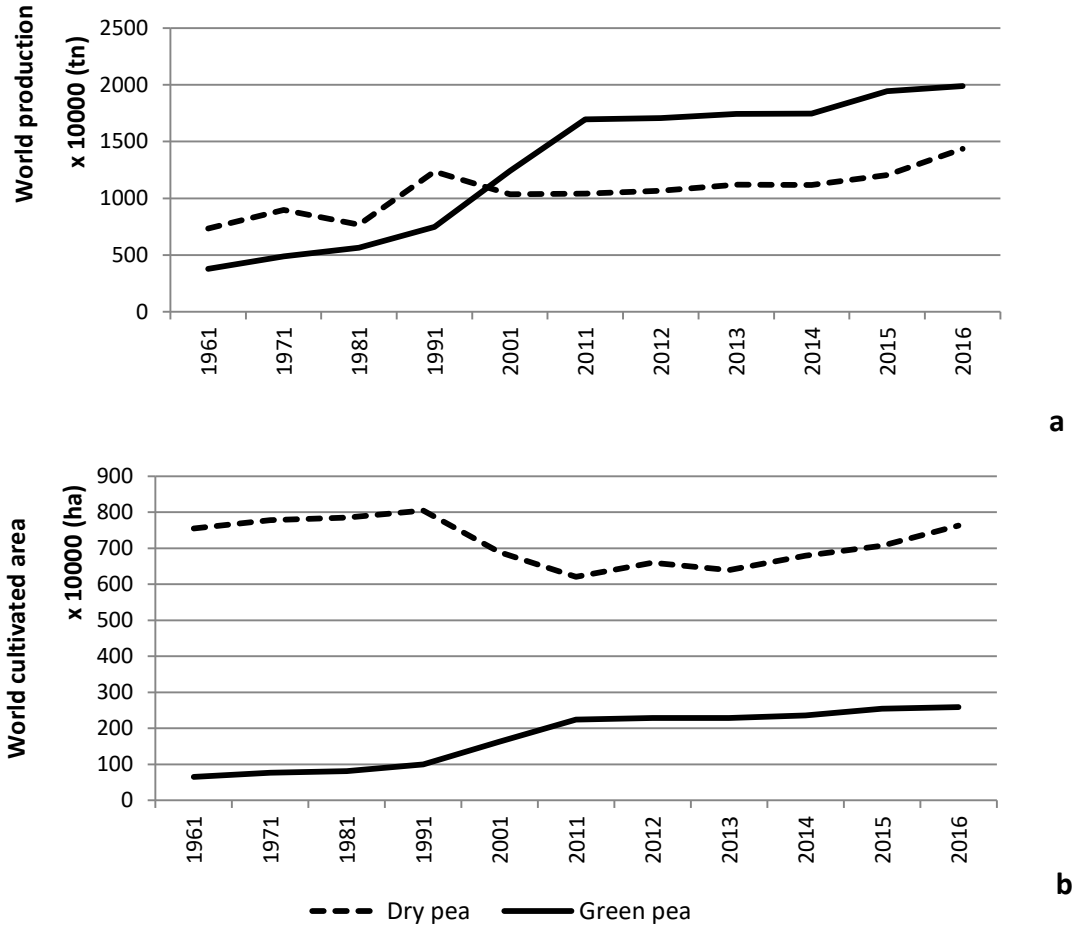

Figure 2. World production (in tonnes) (a); and cultivated area (in hectares) (b), of dry and green pea in from 1961 to 2016 (FAOSTAT).

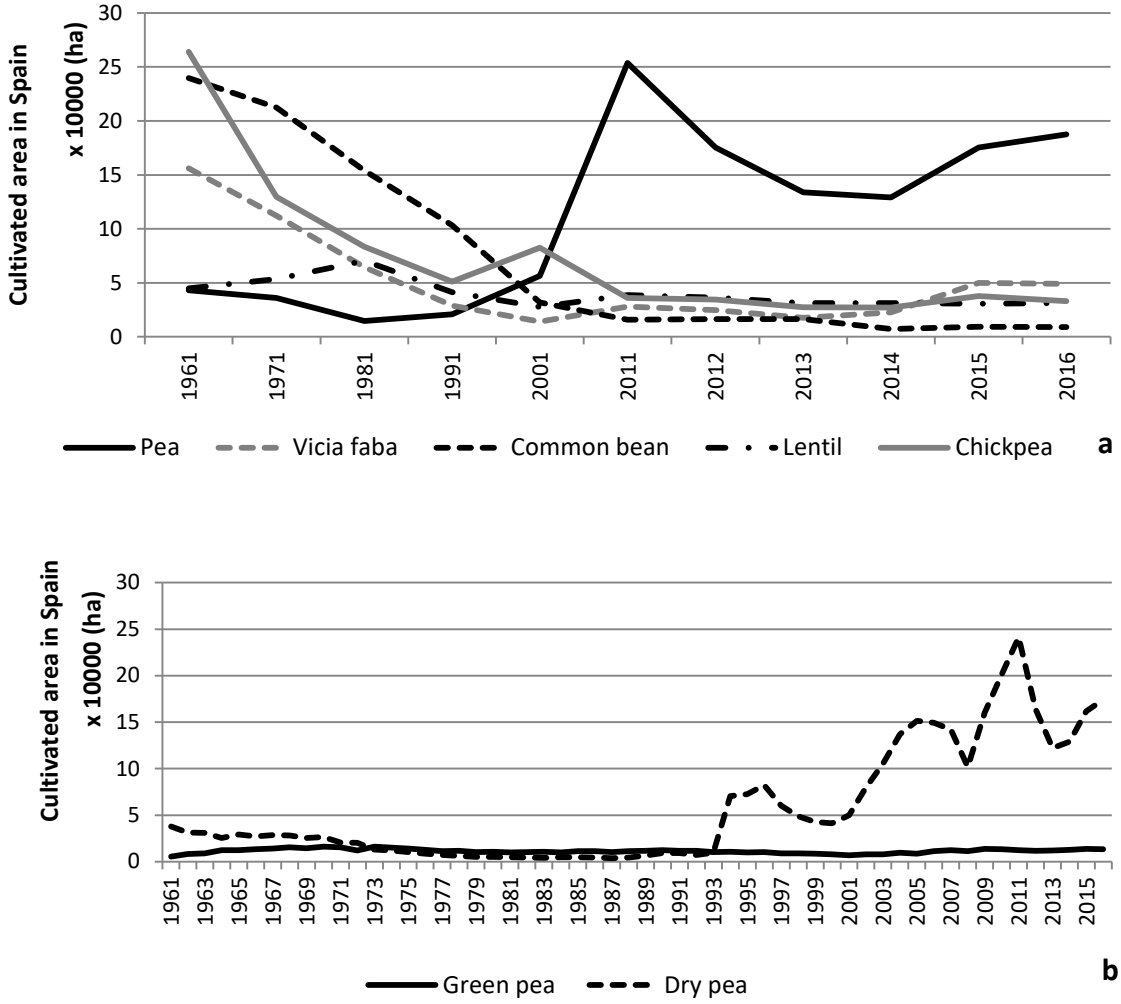

Figure 3. Cultivated area in Spain (hectares) from different pulse crops (a) and in particular dry and green pea crops (b) from 1961 to 2016. (FAOSTAT)

3. BIOTIC STRESSES LIMITING PEA YIELD

Pea yield and quality are heavily diminished by different biotic stresses, such as diseases, pests and parasitic plants. Listed below there are some worldwide problems affecting pea crops.

- Root rots caused by fungus such as *Fusarium solani* f.sp. *pisi* or *Aphanomyces euteiches* (an oomicet), are problematic diseases that usually ends up being associated with diseases caused by other pathogens, causing losses of 60% of the yield (Basu, 1978, Gaulin et al., 2007).

- Vascular disease caused by the fungus *Fusarium oxysporum* f.sp. *pisi*. It's characterized by a sever necrosis and wilting, causing losses that could reach of 80% the yield (Sharma et al., 2006).

- Ascochytosis caused by a fungus complex of *Didymella pinodes*, *Ascochyta pisi* and /or *Phoma medicaginis* var. *pinodella*, that produce chlorosis and necrosis (Rubiales and Fondevilla, 2012).

- Powdery mildew caused by *Erysiphe pisi* fungus, which has aerial dispersion and affects more severally the latest sows (Fondevilla and Rubiales, 2012).

- Rust produced by two different fungus, *Uromyces viciae-fabae* and Uromyces pisi, which by covering pea plants reduce photosynthesis and produces losses of up to 30% the yield (Barilli et al., 2009).

- Pea enation mosaic virus (PEMV) virus that could be spread by several pests and causes necrosis affecting several pulse crops production as chickpea, lentil and beans (Kraft and Pfleger, 2001).

- *Orobanche crenata* Forsk, an important and longstanding parasitic pea plant that could reduce yield an 80% (Rubiales et al., 2005a) by absorbing water and nutrients form the roots.

- Root-lesion nematode (*Pratylenchus* spp.), root-knot nematode (*Meloidogyne* spp.) and pea cyst nematode (*Heterodera goettingiana*) to which little resistance is available (Rubiales et al., 2012).

Below we describe in detail two pests that severely affect worldwide pea crops, and on which this thesis is based.

4. PEA WEEVIL (*Bruchus pisorum*)

Global pea production is spoiled by pea weevil (*Bruchus pisorum* (L.); Coleoptera: Bruchidae), from now on abbreviated as *Bp*. It is an univoltin and strict monophagus insect (Tibor and Szentesi, 2003). When the temperatures reach around

20-25ºC *Bp* emerge from hibernation inside stored grains or in other shelter, being able to fly for several kilometres in order to feed on pea flowers. Once activated, males are ready to mate, instead *Bp* females require fed nectar and pollen (Clement, 1992) to promote oogenesis. Once the females are ready, mate and later on make the oviposition on green filled pods (Hardie and Clement, 2001), process that can suppose 400 eggs for female involving 2-4 weeks (Michael et al., 1990). Depending on weather conditions, around 10 days are needed for the larvae to hatch, goes through the pod wall, penetrates inside a seed and subsequently devours the endosperm in order to moult. This whole process translates into a period of near a month for adult development, however larval development is negatively influenced by accumulated radiation and photoperiod (Aznar-Fernández et al., 2017) (**Chapter1)**.

The *Bp* larval development implying seed consumption, causes an important reduction of yield, while feeding scars and holes on testa decrease the quality and marketability of the seed, as well as are prone to shatter during the harvest (Clement et al., 2002a), in addition to reduce seed germination ratios (Nikolova and Georgieva, 2015) Figure 4.

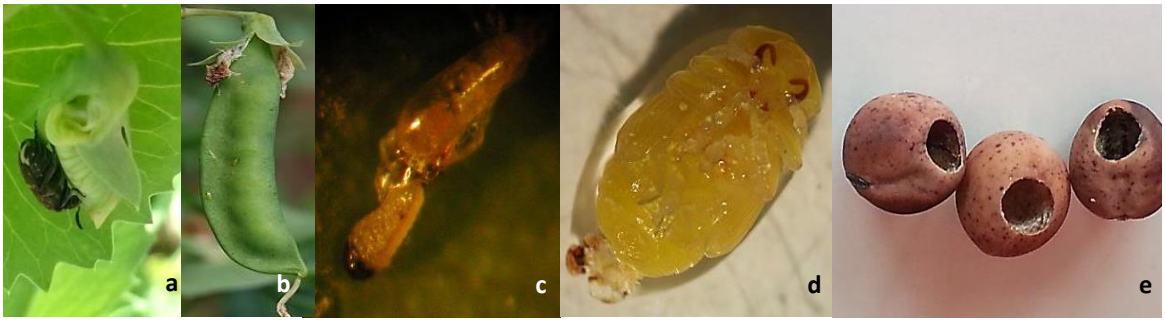

Figure 4. Details of *Bruchus pisorum*: a: adult feeding on pea flower; b: eggs on pea pod; c: first-instar larva hatching from an egg; d: last pupa stage occurring inside pea seed; e: seeds damaged after adult

Due to *Bp* life cycle, the effectiveness of insecticide applications depends on well spray timing to concur with female oviposition (Clement et al., 2009). The larvae must be also sprayed with insecticide before they burrow into the seed pods, or they will be shielded and will continue to damage seed. For all the above and by reason of their long egg lying period (middle spring till early summer) several insecticide applications may be required (Aryamanesh et al., 2013). An added problem is the

complexity to monitor this pest by localizing weevil eggs, which are difficult to detect. Finally in order to prevent next *Bp* generation, post-harvest fumigations are also needed (Clement et al., 2009), causing an extra processing work to remove pea weevil bodies. All this makes the treatment of this pest high economic and ecological cost.

Resistance has been described in *Pisum fulvum* (Pesho et al., 1977, Clement et al., 2002b), with the consequent attempt of introgression in *Ps.* ssp*. sativum* (Byrne, 2005, Aryamanesh et al., 2012) and moderate resistance in *Ps*. ssp. *sativum* (Teshome et al., 2015), *Ps*. ssp. *syriacum* and *Ps*. ssp. *elatius* has been recently described (Aznar-Fernández et al., 2017), **Chapter 1**). Stable reduction in larval development has been also identified in *P. sativum* ssp. *elatius*, *P. abyssinicum* and *P. fulvum* (Clement et al., 2002b, Aznar-Fernández et al., 2017), **Chapter 1**). Studies over interspecific populations between *P. sativum* x *P. fulvum* crosses, highlighted the loss of resistance heritability in pods and propose 3 major recessive alleles (*pwr1*, *pwr2*, *pwr3*) given complete resistance in seed, whereas susceptibility is controlled by three major dominant alleles (*PWR1*, *PWR2* and *PWR3*) (Byrne et al., 2008).

Different bioassays elucidated that lower levels of *Bp* infestation are linked to pod and flower characteristics (Mendesil et al., 2016, Aznar-Fernández and Rubiales, 2018 a); **Chapter 2**) that could be related to volatiles on pea pods (Ceballos et al., 2015) and tannins presence (Barbehenn and Constabel, 2011). In addition, some pea plants showed neoplasm formations as a resistant mechanism to *Bp*, under bruchins effects (*Bp* derived plant regulators (Doss et al., 2000)) jointly with a pisatin up-regulation (Cooper et al., 2005), a phytoalexin associated to *Pisum* defence response (Hadwiger and Tanaka, 2017).

5. PEA APHID (*Acyrthosiphon pisum*)

Aphids are a thoroughly known pest (Dedryver et al., 2010) that dwindle their hosts directly by sucking plants phloem. When aphid colonies spread along the plant remove an important bulk of nutrients and water, nutrient deficiencies and plant stunting which cause important yield and /or quality loses (Maiteki and Lamb, 1985),

(Figure 5 a and d). Indirectly, aphids also act as virus vectors, which is usually the most important damage (Ng and Perry, 2004). In order to be able to nourish properly, aphids showed an obligated bacterial endosymbiont, *Buchnera aphidicola*, that provides riboflavin and essential amino acids to aphids, that are deficient in phloem sap (Douglas, 2006). Of the nearly 100 known species of aphids that have managed to colonize the agricultural environment, one of the most studied and that had become a model system is the pea aphid (*Acyrthosiphon pisum* Harris; Homoptera: Aphididae) (Gao et al., 2008b), from now on abbreviated as PA.

A minimum of 11 distinct sympatric known host races are included in PA, each specialized in a different pulse crop (Friedemann et al., 2015) causing significant damage worldwide (Caillaud et al., 2002, Edwards and Singh, 2006b). The induction of PA specialization has been characterized with quantitative trait loci (QTL) (Hawthorne and Via, 2001, Jaquiery et al., 2012). However, there is still a long way to go in order to understand the mechanisms associated with compatibility among singular aphid and plant genotypes (Goggin, 2007). Secondary metabolites such as plant volatiles (VOC's) play a major role in the early acceptance of a plant as a host (Tuomi, 1992). Subsequently aphids pierce the stylet on the epidermal part. These initial attempting might afford enough information about the surface attributes that possess the plant for the acceptance as a host (Caillaud et al., 2002). Thereafter, Electrical Penetration Graph studies on faba bean, suggest that around 130min are needed for PA to reach the plant phloem (Mutti et al., 2008a). This initial feeding behaviour gives an idea about how would develop the aphid-plant interaction.

The complex life cycle of PA (Moran, 1992, Blackman and Eastop, 2014) with both sexual and asexual reproduction, and their huge phenotypic plasticity for environmental adaptation (Tares et al., 2013, Srinivasan et al., 2014), allow an exponential development and infestation. In addition, the coexistence of three types of pea aphid, those producing winged males or females only, and those producing a mixture of both, award this pest a huge capacity of colonization by flight scattering (Caillaud et al., 2002) making very difficult the pest eradication.

Plants displayed several adaptations in order to prevent or limit aphid infestation such as cell death to limit aphid population (Pegadaraju et al., 2005), chlorosis and /or necrosis (Golowska et al., 2010), being able to be both adaptive to the plant and induced by the aphid (Figure 5 b and c). Examples are the gall formation and leaves curling that provide a sheltered environment for aphids (Figure 5 e). Pea wax quantity also could influence on PA density (Chang et al., 2006). Specific signal molecules generation as defence responses to PA, such as phytohormones, nitric oxid and stable free radicals, have been also reported in pea (Mai et al., 2014). However, both resistant and susceptible plants show a basal defence involving cell wall modification, protein and metabolites with antixenotic (no preference) or antibiotic (interfering over aphid life circle) properties and plant volatiles (VOC's).

Currently several NBS-LRR resistant genes for resistance to other aphids have been identified and cloned. *Mi* from wild tomato confers resistance to potato aphid (Rossi et al., 1998), *Vat* from melon, confers resistance to the cotton/melon aphid and to some viruses (Pauquet et al., 2004). Also the gen *RAP1* in *Medicago truncatula* has been reported (Stewart et al., 2009). Nonetheless, recent studies indicated that host identification and later parturition, result before aphid reach the phloem, what suggest the non-intervention of R-gene-mediated resistance to stimulate or hamper aphid asexual reproduction (Nam et al., 2013). Also technologies such as spectral reflectance are being used on field in order to simplify the management and costs of aphids' pest (Alves et al., 2015).

PA has the capacity to manipulate host plant physiology and metabolism (Goggin, 2007, Carrillo et al., 2013) to benefit themselves prolonging the swallowing of phloem (Carolan et al., 2009). Additionally, the continuous aphid virulence and biotype development (Smith and Chuang, 2014), reinforce the need of a deep understanding of plant-PA interaction. This requirement prompted the characterization of PA resistances mechanism.

Figure 5. Details of pea aphid (*Acyrthosiphon pisum*) infesting pea:. a. Pea pod with an initial colony; b and c: chlorosis produced by pea aphid infestation; d. widespread of pea aphids; e: plants wither.

6. CONTROL MESURES

The use of multiple chemical treatments for both pests is currently the most used method by large-scale farmers, even at the risk of generating resistant aphid clones (Vanlerberghe-Massutti and Guillemaud, 2007). There is the need to develop integrated pest management.

Biocontrol displayed not enough yield efficiency for large-scale farmers due to the several interactions performed between the environment and the diversified community of natural enemies (parasitoids, predators and entomophathogenic fungus) on pea weevil (Huis et al., 1990) and pea aphid (Lagos et al., 2001, Snyder and Ives, 2003). In addition, early sowing, removals of crop residues and intercropping have been studied to control *Bp* (Baker, 1998, Teshome et al., 2016). Also the increase of diversity of the fields has been used to reduce pea aphid infestation levels (Lopes et al., 2015). Nonetheless, those technics did not show enough success for farmers to be competent in the agro-industry food.

On the other hand, there is a growing interest in biopesticides as an alternative method to control many harmful pests and fungus in a more sustainable and economic way (Hynes and Boyetchko, 2006). Several compounds have been described as deterrents or mortals to many fungus and pests (Barilli et al., 2017, Ganassi et al., 2016b) (**Chapter 4).** However the wide range of compounds, pests and diseases make

this recent trend a powerful weapon to be deeply explored in order to control this damaging pests.

For all the above, the use of resistant varieties is considered the most ecological and economical way to combat the diseases and pests of current crops.

7. APPROACHES IN PLANT BREEDING PROGRAMS FOR BIOTIC STRESSES

Several strategies to increase sustainable reintroduction of grain legumes have been developed. Those are mainly focused on breeding production, by identifying and characterizing resistances and devising solutions in term of novel varietal development, culture practices, and food uses.

Germplasm collections are a valuable reservoir of traits that might have been inadvertently eliminated within the cultivated genepool (Smýkal et al., 2012). Therefore, pea breeders are used to look for resistance in wild material and land races showing higher genetic diversity than cultivars, which makes them favourable to be used in breeding programs.

The advent of various molecular marker techniques offer new approaches for improving important agronomic traits, allowing the development of linkage maps, that showed the relative position of genetic regions in the genome, taking into account the recombination frequency of those genetic regions in a segregating mapping population. Flanking markers (Quantitative Trait Loci, QTL) associated to different traits have been developed for several traits such as those for PA resistance, mapping for both: antixenosis and antibiosis mechanism in some species (Meng et al., 2011, Guo et al., 2012) and those associated with cotyledon, pod wall/seed and pod wall resistance for *Bp* (Aryamanesh et al., 2013); **Chapter 3)**. Mapping of interesting genes on the pea genome and the understanding of the interactions of genes involved (mono or polygenic) in the expression of quantitative traits have made possible more efficient and predictable pea breeding programmes.

The study of metabolites and proteins involved in induced or basal plant defence also has allowed the comprehension of pests-plant interactions and the genes implicated, which give new clues to develop or improve breeding strategies (Smith and Boyko, 2007). There are specific studies to battle these pests by the identification of transcriptome sequencing genes differentially expressed and proteins involved, such as for mungbean bruchids (*Callosobruchus* spp.) (Lin et al., 2016) and pea aphid (Carrillo et al., 2013).

Once detected, the introgression of major genes is achieved through backcrossing, pedigree selection and phenotyping. Fixed line selections are grown around 3 seasons under different environments if possible, to permit observations of performance and enable the selection of lines that are more broadly adapted over years and environments. Thereafter, the varieties with better agronomic qualities should be multiply for standards evaluations in order to incorporate them in the marking.

OBJECTIVES

Considering all of the above, the specific objectives of this thesis are detailed below:

1. Identification of novel sources of resistance to *Bruchus pisorum* and *Acyrthosiphon pisum* in a collection of *Pisum* spp. under field conditions (covered in Chapters 1 and 4, respectively).

2. Characterization of the resistance mechanisms occurring in different pea accessions resistant to *Bruchus pisorum* and *Acyrthosiphon pisum* under controlled and/ or semi-controlled conditions (covered in Chapters 2 and 4, respectively).

3. Mapping of QTL involved in low seed infestation and larval development to *Bruchus pisorum* (covered in Chapter 3).

4. Identification of natural compounds that deter *Acyrthosiphon pisum* (covered in Chapter 5).

CHAPTER 1

Identification and multi-environment validation of resistance to pea weevil (*Bruchus pisorum*) in *Pisum* germplasm

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Category: Entomology Sci; JCR: *IF_{2016}=3.73, Ranking 4/91*, **D1**

TITLE:

Identification and multi-environment validation of resistance to pea weevil (*Bruchus pisorum*) in *Pisum* germplasm

ABSTRACT

Pea weevil (*Bruchus pisorum*) is a damaging insect pest affecting pea production worldwide. The aim of this study was to identify sources of resistance to pea weevil in a *Pisum* spp. germplasm in multi-environment field tests. Seed infestation and larval development were assessed in each environment and subjected to a heritability-adjusted genotype and genotype x environment biplot analysis. Results showed that seed infestation and larval development are independent traits. Accessions P669 (*P. sativum* ssp. *elatius*) and P656 (*P. fulvum*) showed a stable reduction of seed infestation across environments. Meanwhile, accessions P314 (*P. sativum* ssp. *elatius*) and P1 (*P*. *abyssinicum*) showed a stable reduction of larval development. The most promising accession was P665 (*P. sativum* ssp. *syriacum)* which showed resistance at both pod and seed levels. This study demonstrates the importance of environmental conditions for pea weevil infestation. Moreover, by submitting data to a CCA, the influence of climatic parameters over seed infestation and larval development has been elucidated.

KEYWORDS: *Bruchus pisorum*, Seed infestation, Larval development, HA-GGE biplot analysis

1. INTRODUCTION

Pea (*Pisum sativum* L.) is the most widely cultivated temperate grain legume in Europe and the second in the world (FAOSTAT, 2014). Pea production is severely affected worldwide by pea weevil (*Bruchus pisorum* L., Coleoptera: Bruchidae), hereinafter referred to as *Bp*, causing losses that can exceed 50% of the harvest (Smith, 1990, Clement et al., 2002b). *Bp* is a univoltine and strict monophagus insect (Tibor and Szentesi, 2003) whose adults present a reproductive diapause during the winter mainly inside stored peas. When peas start to flower, *Bp* adults emerge, feed on flowers, mate and subsequently oviposit on young pea pods (Hardie and Clement, 2001). Around 10 days after the oviposition, larvae hatch and penetrate into the seed, through pod and seed walls, where they devour the endosperm and finally moult. Only one larva develops per seed, but almost all seeds can be infested when pea weevil populations are high; which severely reduces seed yield leading also to quality and marketability depreciation (Brindley and Hinman, 1937, Nikolova and Georgieva, 2015).

Chemical control is possible, although its effectiveness depends on constant monitoring of the field to adjust spray timing to coincide with female oviposition (Horne and Bailey, 1991b, Clement et al., 2009). A single female can produce up to 400 eggs over 2 to 4 weeks (Michael et al., 1990) and repeated insecticide applications are often required (Aryamanesh et al., 2013). Moreover, post-harvest fumigations are required to avoid the emergence of weevils that are hibernating inside infested stored seeds and, consequently, to mitigate the increase in pest level (Clement et al., 2009). This makes treatment of this pest economically and environmentally expensive. On the other hand, integrated pest management has been attempted by introducing biological control with parasitoids (Huis et al., 1990, Barry and O'Keeffe, 1987), or by applying other cultural practices such as early sowing or removal of crop residues by grazing livestock (Baker, 1998, Teshome et al., 2015). However, their effectiveness does not yet fulfil the levels required by the agri-food industry. These difficulties suggest the need to use resistant cultivars.

Only recently, moderate resistance has been described in *Pisum sativum* germplasm (Teshome et al., 2015), but there is still a long way to go to supply commercial needs because there are no cultivars that combine good agronomic traits and useful resistance. This limited availability of resistance prompted the search for genetic resistance in wild relatives of the crop. Resistance has been identified in *P. fulvum* (Pesho et al., 1977, Clement et al., 2002b) and its introgression into *P. sativum* has been attempted (Byrne et al., 2008, Aryamanesh et al., 2012).

Therefore, the aim of this study was to identify and characterize stable sources of resistance to *Bp* in *Pisum* germplasm in different environments by a heritability-adjusted genotype and genotype x environment biplot analysis (HA-GGE) and to elucidate the climatic parameters that most influence the incidence of this pest using a canonical correspondence analysis (CCA).

2. MATERIALS & METHODS

2.1 Plant material, field experimentation and assessments

The studied germplasm collection consisted of 52 *Pisum* spp. accessions (data given in Online Resource, Table 1) including *P. abyssinicum*, *P. fulvum*, *P. sativum* ssp. *arvense*, *P. sativum* ssp. *elatius*, *P. sativum* ssp. *syriacum* and *P. sativum* ssp. *sativum*, maintained at the Institute for Sustainable Agriculture, CSIC, Córdoba, Spain and earlier provided by USDA, USA (PI-numbers), John Innes Centre, UK (JI-numbers), Plant Genetic Resources, Holland (CGN-numbers) or ICARDA, Syria (IFPI-numbers). Accessions were screened in two locations in Spain [Córdoba (CORD): Latitude 37º 51' 25'' N; Longitude 04º 48' 10'' W; Altitude 117m and Escacena del Campo (ESC): Latitude 37º 22' 01'' N; Longitude 06º 32' 29'' W; Altitude 192m] during 3 growing seasons. Some climatic information for each environment assessed is shown in Table 2. Pea cv. Messire was included as the susceptible in all trials.

Experimental fields consisted on 200m^2, with 6 randomized blocks. For each treatment 15 seeds per accession were sown in a single 50cm row and with a separation of 50cm between accessions. Seeds were previously scarified to ensure imbibition and later germination. No pesticides or herbicides were applied on the experimental plots or surrounding fields during the experiments, and they were instead mechanically and manually weeded. Both locations have a known history of high levels of natural pea weevil infestation. Therefore we relied on natural infestation, supplemented with *Bp* adults spreading on the plots (collected from cv. Messire seeds infested during the previous season) when the first adults were observed in the field.

By assessing the flowering of plants in each row weekly at Córdoba, 50% of plant flowering was estimated, and subsequently the number of days to 50% of bloom was determined.

Plants were harvested manually at maturity and threshed; subsequently seeds were stored at 4ºC. Harvested seeds were evaluated for seed infestation (SI) and larval development (LD). For this, 100 seeds per accession and block were randomly selected, placed inside triple vented square Petri dishes with polystyrene lids (PS) (120mm^{2}x15mm) and opened through the cotyledons with the help of a cutter to evaluate larval presence (SI) and larval stage (LS). To soften the seeds, facilitate cutting and prevent the appearance of fungi, a solution of 0,1% NaClO and 1% 8-quinolinol hemisulfate salt (Sigma Aldrich) was applied to the seeds by wetting sterile paper towels placed inside Petri dishes and incubating them at 37ºC for 48h (protocol modified from (Girsch et al., 1999)). In order not to bias the evaluations, all accessions were treated and incubated once the harvest was done, so they were subsequently maintained at 4ºC until SI and LS were assessed; in this way, LD was stopped at all accessions and repetitions. SI (%) was calculated by comparing the number of infested seeds (at any stage of LD) to the total number of seeds evaluated. Once the seeds were opened, LS was assessed using a modified visual scale from (Clement et al., 2002b): LS1= first instar-larval penetration, 0-5% cotyledon eaten; LS2= 6-25% cotyledon eaten; LS3= 26-60% cotyledon eaten, second to fourth instar; LS4= extensive damage, prepupa; LS5= adult (Figure 1). The final larval development index (LD) was assessed using the following formula:

$$LD = \frac{\sum Si\ X\ Ni}{Ne\ X\ Nt}$$

Where *Si* is the larval development stage, *Ni* is the number of seeds with same *Si*, *Ne* is the number of the total stages from the visual scale (5 in our study) and *Nt* is the amount of Ni for each treatment.

Figure 1. Pea seeds of different accessions from the germplasm evaluated, attacked by *Bruchus pisorum* after the solution treatment. A visual scale has been used for the calculation of larval development index (LD). a) First instar penetration, where just a dot is detected, 0-1% eaten, (LS1); b) 2-25% cotyledon eaten, (LS2); c) Second to fourth instar stage, 25-60% cotyledon eaten, (LS3); d) Pre-pupa, extensive damage, (LS4); e) Imago, adult, (LS5)

In order to develop a canonical analysis for SI and LD, a third assessment was required. As such, degree days (ADD) during the period of coexistence of weevils and field peas was determined. The calculation was performed using the amount of available heat above a given threshold temperature (in this study 27ºC, a comfortable temperature for *Bp* in the field) from March to June (when the harvest was completed).

2.2. Statistical analysis

A combined analysis of variance was conducted to determine genotypic differences and genotype x environment interactions for SI and LD. SI data was approximated to normal frequency distribution by means of arcsin square root transformation. Environments were defined as the combination of "cropping season" and "location". Each site in a given year was classified as a separate environment. F-ratios were used to test the effects of the randomized complete block experiments combining location-year environments (McIntosh, 1983). Information of the tested environments is given in Table 2. With the purpose of eliminating interactions between variables and in order to include genotype and genotype x environment (GGE) interactions in the analysis, a HA-GGE biplot analysis was carried out (Yan and Holland, 2010). Biplot graphs are mathematically based on SVD (Singular Value Decomposition) models and are suitable for simultaneous visualization of interacting factors. They are often used when multiple genotypes are compared in different environments (Villegas-Fernández et al., 2009, Sánchez-Martín et al., 2014, Rubiales et al., 2014). In this way, the best genotype would be the one with the lowest values for the evaluated trait and the most stable throughout all the environments; indicating low GxE interactions. This trait will be represented by the proximity to the abscissa from the target environment axis (TEAa) (shown by an arrow with dashed line, indicating the lowest values), which indicates the average performance of all the environments. Around the TEA ordinate (TEAo) the opposite values will be distributed, elucidating the genotypes most affected by the environmental interactions. The length of the environmental vectors will be proportional to the square root of the environmental heritability. Therefore, the shorter the vector, the smaller the differences between genotypes. The angles formed between the environments and the TEAa axis will give an idea of the correlation between them, elucidating the reproducibility and representation of each environment through the cosine of the angles. The acute the angle, the higher the correlation [see Figure 1 in (Flores et al., 2013) for a more detailed explanation].

Pearson correlation was calculated to study the possible relationship between the parameters evaluated. Analyses were performed using SAS® 9.3 (SAS Institute Inc., North Carolina, USA), and the program for graphing GGE biplots was developed by (Burgueño et al., 2003).

To evaluate the influence of environmental factors on SI and LD, different climate variables were subjected to a Non-Metric Multidimensional Scaling (NMDS) ordination (Anderson, 2001). Data on the climatic variables: rainfall, photoperiod (PP), average radiation (Av.Rad) and accumulated radiation (AcuRad) (sum of radiation over all the period), average of minimum and maximum temperature (Av.Tmin and Av.Tmax), as well as average relative humidity (Av.Hum) were obtained from http://www.juntadeandalucia.es/agriculturaypesca/ifapa/ria/servlet/ for each environment. In order to focus on the occurrence of *Bp* in the field, the climatic parameters used in the analysis ranged from March to June. NMDS is a well-suited technique for handling non-normal and non-continuous data (McCune and Grace, 2002) and allows reduction of the matrix of climatic variables before modelling each response variable. Consequently, in order to determine the relative impact of the selected climatic variables on the performance of SI and LD, canonical correspondence analysis (CCA) was carried out. Analysis was performed using Paleontological Statistics Software Package (PAST) (Hammer et al., 2001). The interpretation of the ordination diagram generated is similar to that of the biplot.

3. RESULTS

A wide range of values for SI and LD were noted for the 52 pea accessions studied in the five environments evaluated (see Online Resource, Table 1, where in order to simplify data interpretation relative values are given, which refer to the control (cv. Messire = 100%)). ANOVA (Table 3) revealed a significant effect of genotype (G), environment (E) and GXE in both variables; being the highest mean of square for E, followed by GxE and the lowest for G.

This could be seen in the wide range of SI levels between environments, going from an average of 13.1% for CORD12 to 58.0% for CORD14. In contrast, LD index values encompassed lower differences between the environments, ranging from 51.8 for CORD14 and 71.1 for CORD12.

Table 2. Analysis of variance for *Bruchus pisorum* seed infestation (SI) and larval development index (LD) of the 52 pea genotypes in the five environments evaluated (DF: degrees of freedom; MS: mean square; GxE: term of genotype x environment interaction).

	Source	DF	MS	Variation (%) [a]F
SI	**Environment (E)**	4	100209.5*	58.80
	Replication	25	302.6	
	Genotype (G)	49	1399.4*	10.06
	GxE	196	540.5*	15.54
	Error	955		
	Total	1229		
LD	**Environment (E)**	4	13807.8*	21.91
	Replication	25	99.6	
	Genotype (G)	51	524.8*	10.64
	GxE	204	251.4*	20.35
	Error	986		
	Total	1270		

* Significant at 0.0001 level probability

[a] Fraction of sum of squares associated with each term or interaction

General weather conditions for each environment are shown in Table 2, and their influence over SI and LD elucidated in the CCA diagram (Figure2).

Table 3. Description of the five environments tested during the study (defined as a combination of location and season). The parameters (Tmax, Tmin: absolute maximum and minimum temperatures, respectively; Av. T: average temperature; Av Humidity: average humidity; Rainfall: accumulated rainfall) are given for the period between sowing and harvesting from 2012 to 2014.

Environment	Location	Season	Tmax (ºC)	Tmin (ºC)	Av.T (ºC)	Av. Humidity (%)	Rainfall (mm)
CORD12	Córdoba	2011/2012	37.0	-5.7	13.94	64.68	247.6
CORD13	Córdoba	2012/2013	32.5	-2.0	13.68	76.93	622.6
ESC13	Escacena	2012/2013	32.5	1.0	14.24	74.45	451.6
CORD14	Córdoba	2013/2014	35.0	-3.0	14.03	72.63	522.4
ESC14	Escacena	2013/2014	35.0	2.5	15.15	71.64	376.8

The more influential parameters on SI (closer to SI point in Figure 2) were rainfall, AcuRad and PP. Rainfall had a negative effect, with SI decreasing at rainy seasons as could be seen in the driest environments for CORD12 and ESC14. On the contrary, AcuRad and PP had a positive effect, with SI increasing at higher values. The average of Av.Tmin was less influential but following the same pattern. In contrast, Av.Rad, Av.Tmax as well as Av.Hum had values near the axis centre, which suggests that they have little effect on SI.

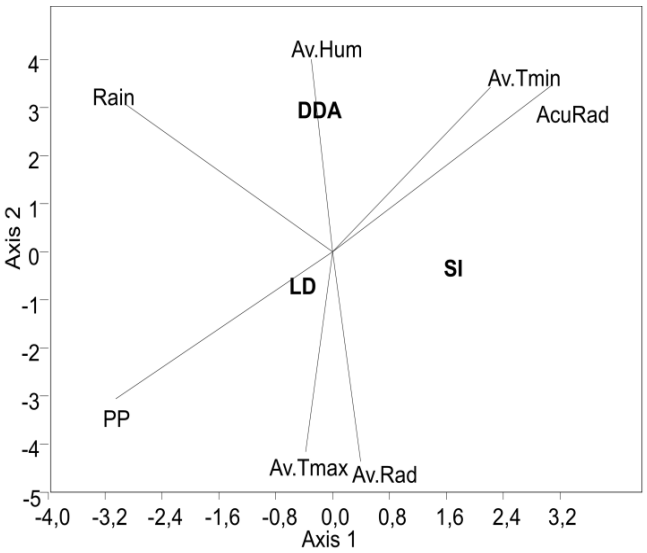

Figure 2. CCA graph based on the correlation of SI, LD and ADD of Bruchus pisorum for 52 pea accessions studied in five environments comprising several climatic parameters. The period analysed was from March to June. PP = photoperiod (min); Av.Hum= average of relative humidity (%); Av.Rad= average of radiation per day (W/m²); AcuRad= accumulated radiation per month (W/m²); Rain (mm); Av.Tmax= average of maximum temperatures (ºC); Av.Tmin= average of minimum temperatures (ºC); SI= seed infestation (%); LD= larval development index; ADD= accumulated degree days (ºC)

Concerning LD, the CCA (Figure 2) showed that the projections of all parameters were close to the centre axis. It should be noted that PP and AcuRad were over LD point, which suggests that these parameters directly affect LD. Both increased LD at lower levels. The CCA also showed, despite projected values of temperature and radiation over the LD being represented close to zero, that a lower Av.Tmax, Av.Tmin and Av.Rad promoted LD, whereas rainfall decreased LD at higher levels.

When analysing the effect of environment on SI and LD for the accessions screened HA-GGE biplots (Figure 3) revealed that environments from CORD formed the smallest angles with the TEAa axis, indicating that it was more representative than ESC. An exception was the year 2014, where vectors from both locations showed similar acute angles in Figure 3a and equal acute angle in Figure 3b.

The HA-GGE biplot for SI (Figure 3a) explained 76% of the information taken from both Principal Components (PC1, PC2) with a relation (G+GE)/(E+G+GE) >10%, which suggests that the HA-GGE biplot provided a robust indication of the interactions (Yang et al., 2009). The environment closest to the origin (indicating lower GxE interactions) was ESC13 followed by CORD13.

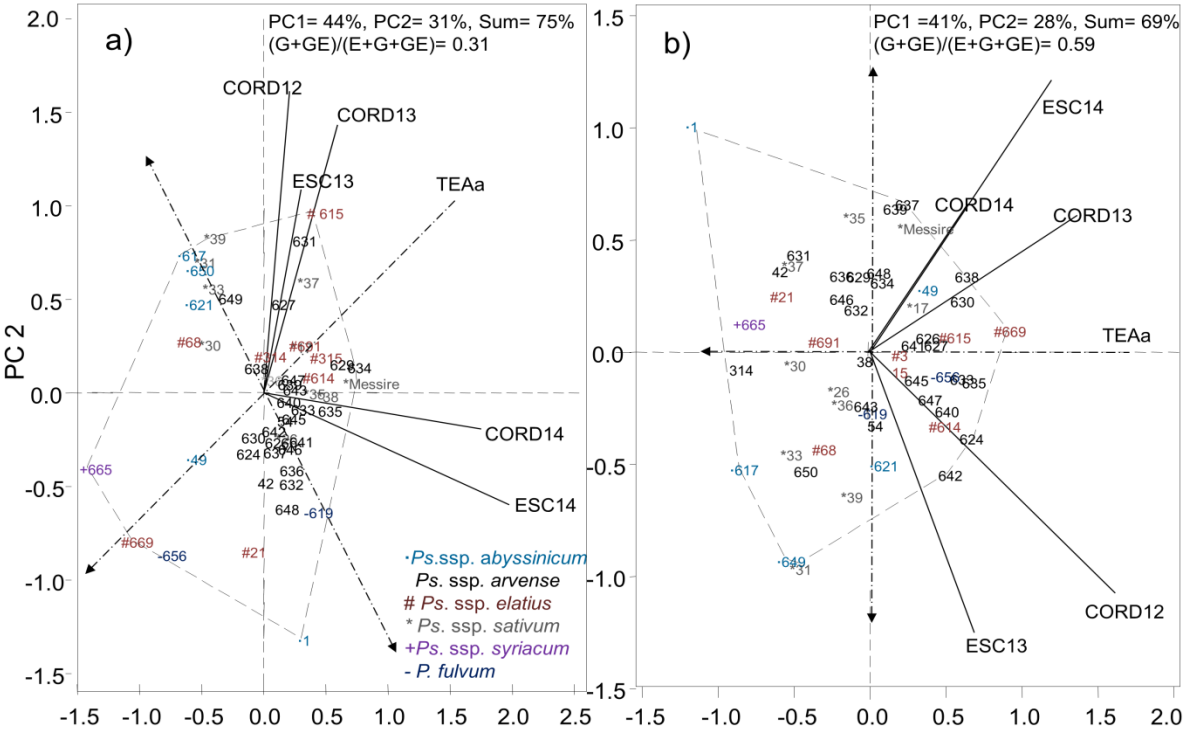

Figure 3. HA-biplots based on (a) *Bruchus pisorum* seed infestation (SI) and (b) larval development index (LD) for 52 pea accessions in the five environments (combination of season x location) studied.

Conversely, CORD14 and ESC14 displayed the longest vectors, which indicated that genotypes showed more differences across each environment, being more discriminative. In order to determine which of the 52 pea accessions studied were the least affected by *Bp* attack based on their representation in the biplots, the ranking of the top ten pea accessions (considering stability across the environments studied) for both variables assessed are shown in Table 4.

Table 4. Ranking of the ten pea accessions with the lowest levels of *Bruchus pisorum* seed infestation (SI) and larval development index (LD). Stability throughout the environments has been taken into account by considering each accession position in the biplots.

	SI	LD
-	P669	314
	P665	P665
	P656	P1
	P49	P617
	P21	P21
	P1	P42
	P68	P649
	P624	P33
	P630	P30
+	P42	P37

Thus, in the case of SI, the accession with the lowest SI was P665 (16%) despite exhibiting environmental interactions, followed by the accessions P669 (26%), P656 (28.4%) and P49 (44.7%), whose responses were more stable, as indicted by their location close to the TEAa axis. The most susceptible accessions (high SI, represented in the opposite side of the biplot) were P315 (94.3%), cv. Messire (100%, control) and P634 (101.4%).

In the HA-GGE biplot based on LD (Figure 3b), PC1 and PC2 explained 69% of information with a relation (G+GE)/(E+G+GE) >10%. This was considered an appropriate method for analysing LD data. The environment with the shortest vector was CORD14 and the longest was ESC14, followed closely by CORD12. Discriminative environments (longer vectors) were those in which less rainfall was registered. The accessions with the lowest values for this trait were P1 (76.3), P314 (85.6) and P665 (86.9) (see Table 4). However, the accession P1 was heavily affected by the environment. On the opposite side of the biplot, displaying the highest LD values, was accession P669 (108.0) followed by P635 (107.9) and P630 (104.8); the latter with environmental interactions.

Pearson correlations between SI and LD with genotype as a weighting variable (r=-0.48, P=0.0001), revealed a significantly low r value, which suggests no association between both parameters. This was also reflected in the arrangement of the accessions in the HA-GGE biplots (Figure 3).

There were significant differences among accessions at precocity, but days to 50% flowering was not correlated with SI values (r= -0.55; P=0.0001) or with LD (r= 0.35; P= 0.0001).

4. DISCUSSION

The assessments carried out to determine SI and LD as a result of *Bp* attack in multiple environments have allowed the identification of *Pisum* accessions with stable resistance. Interestingly, as shown in Figure 3, both the arrangement of the vectors and their length were different in the biplots, suggesting the independence of the variables assessed.

SI is heavily influenced by the environment as weather conditions can affect both the survival and spread of *Bp*, and the growth and precocity of pea plants (Siddique et al., 1999). This can lead to asynchrony between *Bp* appearance in the field and the availability of pea pods affecting *Bp* infestation levels, as suggested by GxE interactions. For SI, the year 2014 was the most discriminative. Regarding the effect of climatic factors on SI (Figure 2) (which can also

be extrapolated to the presence of *Bp* in the field), results suggested that high rainfall and relative humidity limited SI. This might be due to the fact that rainfall might disturb *Bp* oviposition and reduce egg viability (Roubinet, 2016). On the other hand, SI increased with PP. This could determine the end of the diapause, as has been described in other *Bruchus* species (Tran and Huignard, 1992), resulting in an increase of adults in the field and, consequently enhancing the opportunities for oviposition. As the assessments were performed in spring trials, temperatures increased along with day length (PP), which also showed an association with higher levels of infestation as observed in the environments ESC13 and ESC14 (high Av.Tmin) and in CORD12 and ESC13 and 14 (high Av.Tmax). It is therefore difficult to discern the effects of PP from those of increased temperature. On the other hand, PP was also associated with an increase in AcuRad, which had a negative effect on SI, suggesting a detrimental effect of radiation on survival of the eggs and/or larvae. In fact, ionizing radiation is suggested to combat stored insect pests such as pea weevil, and can be used to sterilize individuals (Dioip et al., 1997).

Results highlighted three pea accessions with low and stable SI values in the assessed environments: P665 (*P. sativum* ssp. *syriacum*), P669 (*P. sativum* ssp. *elatius*) and P656 (*P. fulvum*). The reduced SI for these accessions might be the result of the combination of different resistance mechanisms. First, it could be the result of escape from predation caused by the asynchrony between the appearance of the pest and the appropriate stage of the plant to be infested. However, this seemed to have little effect on the germplasm studied since SI level was not related to 50% flowering, which is in agreement with previous studies in pea (Hardie et al., 1995) and lentil (Laserna-Ruiz et al., 2012). Nonetheless, some resistant accessions (P656 and P669) flowered later than the average, which could have contributed in some way to the escape of these pea plants from *Bp* infestation. In addition, antixenosis mechanisms might be involved in the resistance of these accessions by reducing the preference of pea weevil adults for feeding in their flowers or to oviposit over their pods as the result of morphological or chemical plant factors that adversely affect the insect behaviour (Pouzat, 1981, Bruce et al., 2011, Ceballos et al., 2015). Furthermore, a battery of antibiosis mechanisms could have been triggered in response to *Bp* attack. The reduced values of SI in these accessions could be related to the presence of morphological traits that hinder the penetration of the larvae, such as pod and/or seed coat thickness, e.g. in P665 and P656 (Figure 4b and g); chemical compounds that hamper the penetration of pods and/or seeds; plant volatiles that stimulate parasitism (Paré and Tumlinson, 1999) or wound response of the plants (Bennett and Wallsgrove, 1994). Possible evidence of antibiosis in the accessions

studied could be the presence of purple pigmentation on the pods and seeds of accessions P314 and P665, characterized by low LD and low SI, respectively (Figure 4.a and b). This pigmentation could be produced by condensed tannins (Wang et al., 1998), which are known to be associated with plant insect defence (Barbehenn and Constabel, 2011, War et al., 2015) and could be involved in pea weevil female preference at egg laying (Stam et al., 2014) or even be toxic to the *Bp* larvae at the pod or seed coat level (Figure 4d, e) (Boughdad et al., 1986). It has been suggested that seed colour could be related to the level of resistance to *Bp* (Aryamanesh et al., 2012; Teshome et al., 2016) independently of the genotype, so that the creamy seeds appear to be more damaged than green ones. Although the colour of the seeds was not evaluated in this study, it was observed that some accessions with green seed coat pigmentation exhibited higher levels of susceptibility to *Bp* attack such as P38 (*P sativum* ssp. *sativum*), P627 and P631 (*P. sativum* ssp. *arvense*).

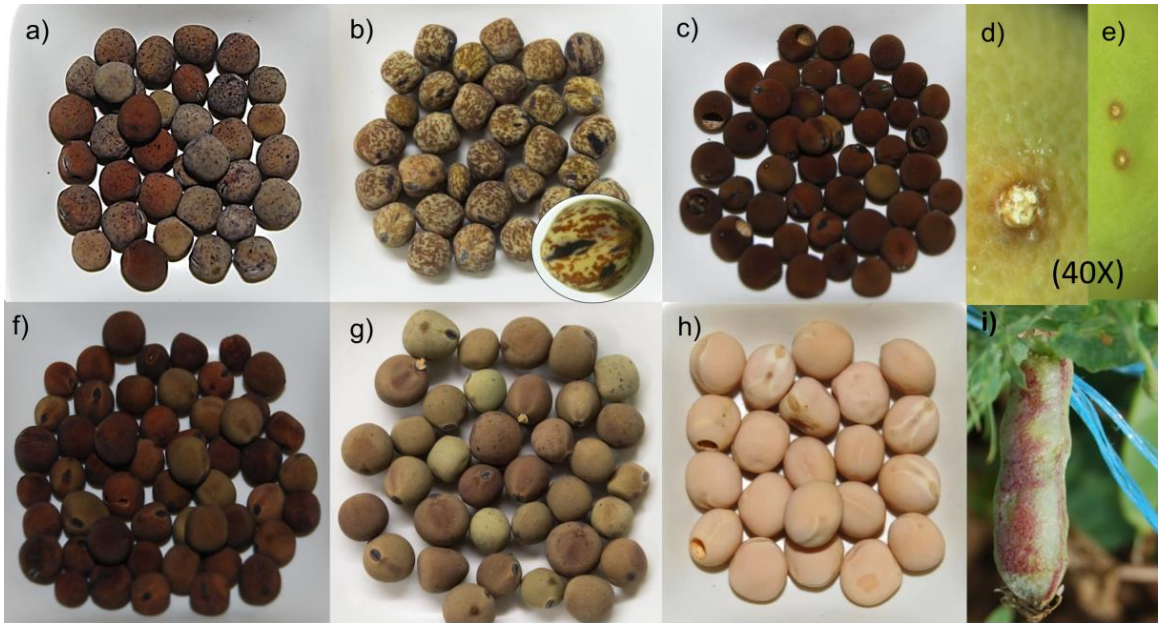

Figure 4 Seeds of the genotypes evaluated in the field showing low values of seed infestation (SI) and larval development index (LD) (a-c and f-h), as well as tannins pigmentation on pods of a resistant accession (b) and a demonstration of *Bruchus pisorum* larvae penetration of a seed (d) and a pod (e). a) P314 accession characterized by low LD; b) P665 accession characterized by low SI and LD; c) P1 accession characterized by low LD, showing *B. pisorum* adult exit holes; d) and e) inlet holes of *B. pisorum* larvae on susceptible genotypes from the collection studied (40X); f) P669 accession characterized by low SI; g) P656 accession characterized by low SI; h) Messire (control) showing exit holes of *B. pisorum* adults; i) pea pod of the accession P665, showing purple pigmentation.

Another antibiosis mechanism acting on *Bp* resistance in the germplasm studied was callus formation on the pods, as was seen in the accession P669 (Figure 5a, b, c and e). This mechanism, described as neoplasm formation (Doss et al., 1995a, Doss et al., 2000), might

prevent *Bp* infestation by hindering the egg permanence on the pea pods. The presence of callus could also have forced the larvae to find a clean site for penetration, exposing them to desiccation, predators and to the detachment of the pod (Clement et al., 2009, Mendesil et al., 2016).

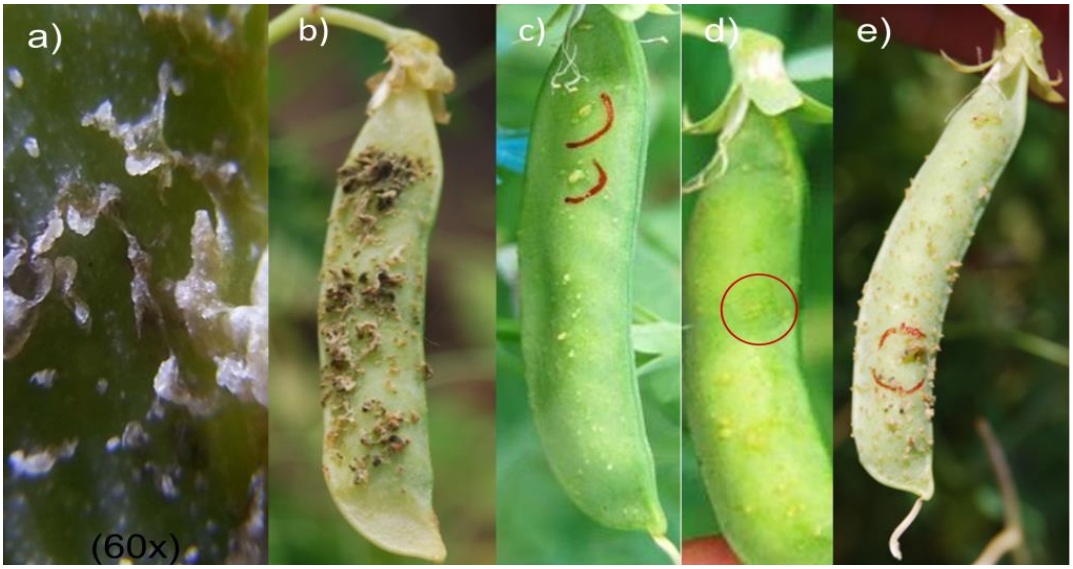

Figure 5 Pods from accession P669 (*Pisum sativum* ssp. *elatius*) under field conditions. (a) Neoplasm formation (60X); (b) neoplasm formation without *Bruchus pisorum* eggs; (c) neoplasm formation under *B. pisorum* egg, (highlighted in red); (d) *B. pisorum* egg without neoplasm formation, surrounded in white; (e) neoplasm formation under *B. pisorum* eggs on the last phase (surrounded in red), where it is expected that the thickness of the neoplasm prevents the entry of the larva through the pod.

Neoplasm development is linked to the expression of the wild-type allele *Neoplastic pod* (*Np*), which causes undifferentiated cell growth. This can be induced by environmental factors such as light quality (absence of UV light) or bruchins production (a plant defence elicitor stimulated by *Bp* contact that induces neoplasm formation beneath the egg (Doss et al. 2000)). Neoplasm formation was also exhibited by other accessions in the collection, being more frequently observed in the greenhouse (data not shown), as also reported by (Teshome et al., 2015). This fact supports our findings under field conditions, where the presence of this trait was modest and/or inconsistent (Doss et al. 2000; Teshome et al. 2016), indicating that the environment should play a major role in their development. Nonetheless, in our field trials, the accession P669 also showed neoplasm in the absence of pea weevil eggs and, conversely, some eggs without neoplasm underneath were observed (Figure 5c and d).

Once the seeds are infested, antibiosis mechanisms might operate by constitutive or *de novo* synthesized metabolites that are toxic or harmful to the insects, delaying or

preventing *Bp* growth inside pea seeds, as evidenced by reduced LD. In addition to this, the nutritional quality of the seeds might also affect *Bp* survival if nutritional requirements of the larvae are restricted. Seed quality could be influenced by both genetics (ability to take advantage of environmental conditions) and environmental factors (effect of the environmental conditions of the crop on the nutritional quality of the seeds) (Berger et al., 1999). This might explain the high importance of GxE interaction on LD (Table 3). Thus, the CAA analysis showed that most of the climatic parameters evaluated were close to the centre of the axis (Figure 2), supporting the theory of a determinant role of nutritional quality of the seed in LD. However, low PP, AcuRad and minimum temperature values directly influenced LD of *Bp*. It is known that photoperiod-temperature interaction is an important factor promoting pea flowering at high levels (Berry and Aitken, 1979) and it seems that this interaction could also be important for *Bp* LD, because when both parameters reached low values, LD was increased. These conflicting results between pea flowering and LD values suggest that the costly investment of energy and resources that pea plants require to flower and produce pods might diminish nutrient availability, which directly affects *Bp* LD.

Low values of AcuRad were associated with increased LD. (Saradhi et al., 1995) reported that UV radiation promotes lipid peroxidation and induces proline accumulation in plants, which would not only decrease seed quality or even damage them, but also could affect the feeding of *Bp*. This synthesis of different response metabolites in pea seeds that could indirectly prevent *Bp* development could be extended to other biotic and abiotic stresses that affect the crop. For example, drought in the field could damage the photosynthetic pathway, which causes alterations in the normality of the redox state of the cells and generates toxic effects (Iturbe-Ormaetxe et al., 1998). The metabolic response of plants at CORD12 and ESC14, the driest environments, may explain why they were the more discriminative ones (Figure 3b). Unfavourable environmental conditions tend to restrict and select against pea weevil LD. Moreover drought also decreases the content of soluble sugars and proteins including α and β-amylases (Al-Jebory, 2012), both involved in starch digestion and therefore, under low values could limit *Bp* LD. In fact, genetically modified peas resistant to *Bp* have been developed by incorporating the insecticidal activity of the α-amylase inhibitor, which is present in bean seeds (*Phaseolus vulgaris* L.) (Schroeder et al., 1995, Sousa-Majer et al., 2007). However, (Prescott et al., 2005) showed that the transgenic expression of the α-amylase inhibitor results in the synthesis of a protein variant with altered immunogenicity in mice, which compromises its practical use.

In order to consider GxE interactions, a HA-GEE biplot analysis was performed (Figure 3b), revealing three interesting pea accessions with low larval development values. The accession with the lowest LD, but holding important environmental interactions, was P1 (*P. abyssinicum*), with dark seeds. Other accessions with low LD values but higher stability among environments were: P314 (*P. sativum* ssp. *elatius*), presenting variegated seed coats; followed by P665, which, as described above, also presented variegated seed coats. In this respect, pea coloured seed coats have been described to have a higher content of free phenolic acids compared to clear-seeded types and have a considerable amount of condensed tannins (these compounds are not present in white seed coats) (Troszynska and Ciska 2002). As described above, tannins have been reported to affect LD of *Callosobruchus maculatus* (Boughdad et al., 1986) and flavonoids affecting *C. chinensis* (Salunke et al., 2005). Thus, in order to identify seed components involved in hindering LD, metabolomic and proteomic studies have been initiated and will be presented elsewhere.

Furthermore, the result of no relation between SI and LD suggests that *Bp* females might not be capable of discerning the suitability of the seeds for LD during the oviposition.

According to the results above, P665 seems to have a clear advantage in defending itself from *Bp* attack. Its low SI and LD makes it particularly interesting for breeding purposes because it probably presents a combination of different mechanisms acting in a pyramidal sense: 1) preventing larval penetration and; 2) in the case of perforation, hindering or preventing the development of larvae. The possibility of combining these two types of resistance mechanisms in elite germplasm is of great importance because the durability of the resistance lies in the availability of different sources of it; thus, the plant will continue to have defence barriers to successfully overcome an attack if one of these levels is broken. Furthermore, the introgression of post-penetration (at both pod and seed levels) resistance is especially important in order to prevent secondary infestations and reduce market depreciation. A high added value is that P665 has been reported as resistant and moderately resistant to different stresses such as pea aphid (*Acyrtosiphon pisum*) (Carrillo et al., 2013), ascochyta blight (*Mycospharella pinodes*) (Fondevilla et al., 2005), broomrape (*Orobanche crenata*) (Rubiales et al., 2005b) and drought (Iglesias-García et al., 2015). Therefore, this study further highlights the usefulness of this accession in pea breeding.

This multi-environment study has identified sources of resistance to *B. pisorum* attack based on antibiosis and antixenosis mechanisms that prevented pea SI and LD. This is the first time that pea weevil resistance has been identified in *P. sativum* ssp. *elatius* and ssp. *syriacum*

germplasm and it is important to point out the low levels of SI that these accessions have presented in relation to the other genotypes studied and the different environments assessed. No complete resistance was identified in any accession and complex inheritance was suggested; however, genetic analyses are needed before this can be stated. With the new information provided, the challenge would be to understand the overall mechanisms involved in the response of peas to *Bp* infestation, as well as to accelerate the identification of specific resistance targets. The purpose would be to implement this in plant breeding programs and to transfer this resistance to elite cultivars in order to achieve a more sustainable crop management. In addition to the new stable sources of resistance against *Bp* infestation reported, this study also suggests that pea weevil females are not able to discern seed suitability for larval development during oviposition. Furthermore, according to our knowledge, this is the first report about the most influential climatic factors in the field over SI and LD, which broadens the information on the biology and preferences of this damaging pest.

5. ACKNOWLEDGMENTS

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Table 1. List of *Pisum* accessions evaluated with mean for each environment for seed infestation (SI) and larval development index (LD) and general mean for environment, are given relative to the control (cv. Messire). Environment names correspond to those defined in Table 2

				SI Mean relative to the control					LD Mean relative to the control				
Accession	**Synonym**	**Species**	**Subspecies**	**CORD12**	**ESC13**	**CORD13**	**ESC14**	**CORD14**	**CORD12**	**ESC13**	**CORD13**	**ESC14**	**CORD14**
cv. Messire	(control)	*sativum*	*sativum*	100 (17)*	100 (17.83)*	100 (23.33)*	100 (74.04)*	100 (89.64)*	100 (65)*	100 (64.51)*	100 (75.32)*	100 (70.29)*	100 (56.29)*
P1	PI 358615	*abyssinicum*		0.00	0.00	30.00	92.69	88.92	49.23	68.72	78.54	91.59	93.53
P17	PI 344003	*sativum*	*sativum*	86.29	105.44	112.86	71.18	81.51	108.74	112.14	84.16	103.49	91.90
P21	PI 505059	*sativum*	*elatius*	47.06	19.07	29.28	75.51	59.63	89.23	85.98	80.99	86.87	93.50
P26	PI 116056	*sativum*	*sativum*	36.76	78.52	102.87	77.74	66.95	104.94	110.26	77.20	91.44	87.74
P30	PI 242027	*sativum*	*sativum*	83.35	94.39	95.71	40.33	41.62	94.60	99.89	79.41	87.54	83.94
P31	PI 269762	*sativum*	*sativum*	152.94	78.52	104.59	33.28	40.94	121.03	115.75	78.66	72.04	88.91
P33	PI 505062	*sativum*	*sativum*	93.53	148.63	98.59	38.51	41.13	87.69	123.87	81.64	83.68	78.74
P35	PI 343987	*sativum*	*sativum*	69.12	84.13	110.72	75.88	87.76	101.05	75.36	96.53	92.26	96.77
P36	PI 343988	*sativum*	*sativum*	92.65	63.54	100.30	66.91	64.95	115.38	103.70	75.12	90.84	85.68
P37	PI 505080	*sativum*	*sativum*	128.82	87.49	145.74	80.77	67.61	90.26	80.49	85.99	87.01	96.39
P38	PI 505092	*sativum*	*sativum*	149.00	61.69	59.28	96.91	83.09	110.75	98.62	98.13	83.75	97.71
P39	PI 505111	*sativum*	*sativum*	170.59	84.13	97.86	18.00	63.39	119.48	115.51	88.86	80.79	81.36
P42	PI 268480	*sativum*	*arvense*	72.53	30.85	60.01	79.84	59.78	78.46	91.97	84.01	90.25	89.13
P49	JI 227	*abyssinicum*		72.53	9.81	61.08	41.19	39.05	99.49	120.14	96.26	101.38	98.42
P54	JI 804	*sativum*	*arvense*	11.76	90.69	132.15	74.59	66.40	123.08	105.96	89.29	86.19	88.95
P68	IFPI 3330	*sativum*	*elatius*	54.88	125.24	97.86	31.54	32.08	134.80	83.71	76.73	80.17	88.56
P314	CGN10205	*sativum*	*elatius*	112.76	22.43	121.43	55.61	71.76	75.38	104.72	77.20	82.47	88.31
P315	CGN10206	*sativum*	*elatius*	35.29	121.14	154.31	84.41	76.46	117.60	105.88	94.03	92.36	94.30
P614	IFPI 3365	*sativum*	*elatius*	54.88	143.97	102.14	81.09	80.51	142.48	106.92	90.43	89.47	100.27
P615	IFPI 3370	*sativum*	*elatius*	163.24	98.15	163.74	75.92	74.19	117.78	116.31	96.32	100.28	90.46
P617	IFPI 387	*abyssinicum*		103.94	123.39	118.95	22.18	33.84	83.08	114.02	83.96	74.15	80.60
P619	IFPI 411	*fulvum*		84.29	25.24	49.29	105.06	78.95	123.83	100.02	89.72	84.27	93.30
P621	IFPI 436	*abyssinicum*		85.29	109.93	110.72	29.21	39.00	114.82	124.82	85.89	88.78	83.55

P624	IFPI 2348	*sativum*	*arvense*	25.47	59.84	97.86	58.28	63.55	139.46	123.40	95.37	90.72	108.72
P626	IFPI 2350	*sativum*	*arvense*	29.41	107.68	81.44	66.27	73.95	132.14	92.15	96.87	93.40	90.25
P627	IFPI 2351	*sativum*	*arvense*	116.18	104.32	117.15	64.56	69.87	118.58	114.03	91.73	98.42	95.19
P629	IFPI 2353	*sativum*	*arvense*	102.94	95.34	112.17	100.19	81.95	103.55	88.03	93.68	92.06	92.17
P630	IFPI 2354	*sativum*	*arvense*	40.00	89.74	75.74	51.32	72.59	117.85	111.91	98.41	102.29	93.75
P631	IFPI 2355	*sativum*	*arvense*	164.71	60.74	156.88	67.34	77.31	84.62	84.13	94.28	85.50	94.21
P632	IFPI 2356	*sativum*	*arvense*	35.29	50.48	83.58	74.11	81.26	115.38	80.88	94.04	87.10	93.18
P633	IFPI 2357	*sativum*	*arvense*	86.29	55.13	95.03	69.71	89.97	132.94	113.30	93.77	97.33	93.75
P634	IFPI 2358	*sativum*	*arvense*	125.00	80.37	104.29	106.37	91.05	90.58	110.64	96.97	96.63	92.06
P635	IFPI 2359	*sativum*	*arvense*	46.06	114.41	109.73	93.19	81.93	139.20	110.71	95.51	95.99	97.89
P636	IFPI 2360	*sativum*	*arvense*	29.41	82.73	84.31	83.55	69.12	71.32	117.54	94.07	95.82	90.99
P637	IFPI 2361	*sativum*	*arvense*	23.53	52.33	112.17	64.13	73.90	103.22	89.58	96.24	100.03	103.30
P638	IFPI 2362	*sativum*	*arvense*	64.71	122.43	94.30	61.08	57.26	117.22	109.67	97.49	102.50	103.57
P639	IFPI 2363	*sativum*	*arvense*	96.06	61.69	101.16	72.41	74.00	98.46	92.30	93.76	103.27	90.03
P640	IFPI 2364	*sativum*	*arvense*	54.41	74.31	117.15	71.85	72.14	130.98	117.51	92.55	94.45	92.41
P641	IFPI 2365	*sativum*	*arvense*	39.24	124.79	72.14	78.47	80.69	129.06	96.86	86.10	94.99	96.55
P642	IFPI 2366	*sativum*	*arvense*	48.53	97.20	76.43	60.58	78.09	134.78	125.55	97.69	87.59	94.97
P643	IFPI 2367	*sativum*	*arvense*	68.24	131.80	81.44	76.19	74.00	112.12	109.62	92.34	86.23	86.62
P645	IFPI 2369	*sativum*	*arvense*	86.29	93.94	66.01	82.69	69.17	114.95	119.28	89.38	96.53	89.70
P646	IFPI 2370	*sativum*	*arvense*	97.06	77.57	46.29	87.12	65.82	88.05	102.14	97.53	90.61	86.55
P647	IFPI 2371	*sativum*	*arvense*	129.41	74.76	70.00	81.36	70.28	123.09	114.05	101.46	87.94	98.38
P648	IFPI 2372	*sativum*	*arvense*	28.24	43.47	77.15	83.32	70.09	109.62	92.99	81.73	100.40	92.20
P649	IFPI 2441	*abyssinicum*		101.00	98.15	125.72	54.70	37.00	110.23	123.91	74.58	75.34	84.30
P650	IFPI 2495	*abyssinicum*		132.35	116.83	91.43	31.36	37.93	102.34	112.96	89.79	78.25	79.27
P656	IFPI 3250	*fulvum*		24.53	33.65	9.99	45.95	27.70	138.80	96.90	99.58	90.30	93.50
P665	IFPI 3280	*sativum*	*syriacum*	17.65	17.95	36.43	2.70	5.21	85.12	86.81	77.89	79.67	104.81
P669	IFPI 3330	*sativum*	*elatius*	47.06	9.37	25.72	21.57	26.03	149.35	105.67	85.36	109.76	89.91
P691	unknown	*sativum*	*elatius*	103.94	107.68	106.43	82.27	55.50	95.89	98.56	87.40	87.44	86.21
			Environment Mean:	77.39 (13.16)*	79.71 (14.21)*	92.07 (21.48)*	66 (48.99)*	64.74 (58.04)*	109.46 (71.15)*	103.96 (67.06)*	89.51 (67.42)*	90.8 (63.82)*	92.04 (51.81)*

	SE	6 (1.02)*	5.12 (0.91)*	4.62 (1.07)*	3.34 (2.47)*	2.75 (2.47)*	2.87 (1.86)*	1.93 (1.24)*	1.05 (0.79)*	1.13 (0.79)*	0.88 (0.5)*

*Actual values of SI and LD for Messire are in brackets, as well as for environment mean and SE.
Accessions included in this table were those with at least tree replicates per environment.

CHAPTER 2

Source of flower and pod influence on pea weevil (*Bruchus pisorum*) oviposition capacity and preference

Aznar-Fernández T, Rubiales D, 2017. Influence of flower and pod source on pea weevil (*Bruchus pisorum*) oviposition capacity and preference. En evaluación en Annals of Applied Biology.

TITLE:

Source of flower and pod influence on pea weevil (*Bruchus pisorum*) oviposition capacity and preference

ABSTRACT:

Bruchus pisorum is an insect pest causing major damage to pea seeds worldwide. Adults feed on pollen, lay eggs on pods and the larvae feeds into pea seeds. Control is difficult and no resistant cultivars are available. In this work we studied possible effects of pollen and pod source on insect fecundity and oviposition by comparing resistant and susceptible *Pisum* accessions and non-host (*Lathyrus sativus* and *Vicia faba*) species. No choice assays revealed that the source of flower offered to adults for feeding might retard oviposition (the case of *V. faba*), reduce fertility (*P. sativum* ssp. *syriacum*, P. *fulvum* and *V. faba*) or increase adult mortality (*V. faba* and *P. sativum* ssp. *syriacum*). A second no-choice assay with all adults fed with pollen of the same pea cultivar showed significant effect of the source of pods offered for oviposition, being reduced in some resistant *Pisum* accessions, but particularly low on pods of the non-hosts, being retarded if ever happening, coupled with high mortality of adults. This was confirmed in a third experiment consisting on dual-choice assays showing reduced egg lying in *V. faba*, *L. sativus*, *P. fulvum* and *P. sativum* ssp. *syriacum* compared to the check pea cv. Messire.

KEYWORDS: *Bruchus pisorum*; oviposition; antixenosis; deterrence; preference

1. INTRODUCTION

Field pea (*Pisum sativum* L.) is an important temperate grain legume, ranking first in acreage in Spain and Europe, and second at world level (González-Bernal and Rubiales, 2016b). Their use extends to dry peas for animal fodder and green peas for human consumption. In addition, as a legume, it brings environmental benefits being a safe bet for sustainable agriculture (Rubiales and Mikic, 2014).

As most crops, pea can be constrained by a number of pests and diseases, being pea weevil (*Bruchus pisorum* L., Coleoptera: Bruchidae, *Bp*) a serious concern worldwide. *Bp* is a specialized pest that may cause yield losses of up to 50% (Clement et al., 2002a, Keneni et al., 2011). After a period of hibernation, *Bp* females feed on pollen and oviposit on pods. Once the egg has hatched, the emerged larvae penetrate through the pods to the seeds, where they feed by eating the endosperm (Teshome et al., 2015). This reduces seed yield and devaluates seed quality and marketability (Brindley and Hinman, 1937, Nikolova and Georgieva, 2015). Effective chemical control would require repeated treatments at flowering and fruiting (Michael et al., 1990, Horne and Bailey, 1991a) coupled with post-harvest fumigations in order to prevent adults emerge inside storehouse (Clement et al., 2009). Biological control (Huis et al., 1990) and management by intercropping (Teshome et al., 2016) have been attempted with no definitive results. Use of resistant cultivars would be most recommended but they are not available so far, although some genetic resistance has been reported in pea germplasm (Teshome et al., 2015{Aznar-Fernández, 2017 #138)). Availability of unattractive or repellent genotypes for oviposition would help in designing crop mixtures to manage the pest (Ratnadass et al., 2012, Shelton and Badenes-Perez, 2006) and in breeding resistant cultivars.

The influence of pod characteristics on *Bp* oviposition has been recently elucidated (Mendesil et al., 2016). Pollen ingested might also influence *Bp* dispersal behaviour, longevity, (pre)oviposition period and fecundity (Wäckers et al., 2007). In order to further understand host and non-host plant genotypic effects on sexual maturity of adults and on oviposition preference, we studied the effect of flowers and pods of susceptible, resistance and non-host accessions on female sexual maturity and oviposition capacity, and the effects of their pods on oviposition preference.

2. MATHERIAL & METHODS

2.1. Field screenings

Thirteen pea accession showing different levels of *Bp* infestation were selected from a previous work (Aznar-Fernández et al., 2017) and were field screened during 2014/15 season at Córdoba and at Escacena, Spain (Table 1). The experimental design consisted on a complete block design with 3 randomized repetitions. Each accession was represented by 25 seeds planted in a 50 cm long row, with a separation of 50 cm between accessions. Córdoba's trial was drip irrigated whereas Escacena's trial was rain fed. No pesticides neither herbicide were applied and only mechanical weeding was done when needed. When natural *Bp* infestation was first observed in the area, *Bp* adults were spread on the plots at the rate of 3-4 adults/m^2. These have been collected from cv. Messire seeds infested during the previous season and stored at 4ºC.

Table 1. Pea weevil seed infestation percentage (%SI) of 13 *Pisum* accessions under field conditions (2014-2015).

					%SI ± SE	
Accession	**synonym†**	**Origin**	**Species**	**Subspecies**	**Cordoba**	**Escacena**
P26	PI 116056	India	*P. sativum*	*sativum*	44.7± 6.4	40.3± 0.3
P36	PI 343988	Turkey	*P. sativum*	*sativum*	34.6± 1.4	29.7± 2.7
P37	PI 505080	Cyprus	*P. sativum*	*sativum*	49.2± 1.2	27.7± 8.2
P38	PI 505092	Cyprus	*P. sativum*	*sativum*	42.9± 7	23.6± 4
P39	PI 505111	Syria	*P. sativum*	*sativum*	38.8± 5.4	18.8± 9.8
P624	IFPI 2348	Ethiopia	*P. sativum*	*arvense*	48.1± 1.7	22.7± 1.8
P638	IFPI 2362	Ethiopia	*P. sativum*	*arvense*	43.3± 1.8	30.2± 1.2
P639	IFPI 2363	Ethiopia	*P. sativum*	*arvense*	47.3± 7.2	32.7± 12.9
P646	IFPI 2370	Ethiopia	*P. sativum*	*arvense*	40.6± 2.7	41.0± 5.3
P656	IFPI 3250	Syria	*P. fulvum*		22.5± 3.8	22.5± 3.8
P665	IFPI 3280	Syria	*P. sativum*	*syriacum*	5.6± 3.1	2.1± 0.4
P669	IFPI 3330	Turkey	*P. sativum*	*elatius*	16.0± 3.8	7.0± 1.2
Messire		France	*P. sativum*	*sativum*	81.7± 3.5	45.2± 3.6
				Location Mean ± SE:	39.6 ± 3.0	26.41 ± 2.3
				df:	12	12

† PI-numbers: accessions provided by USDA, USA; IFPI-numbers: accessions provided by ICARDA, Syria.SE, standard error.

At maturity, seeds were manually harvested, threshed and assessed for seed infestation (SI) by opening 100 seeds of each repetition through the cotyledons (Aznar-Fernández et al., 2017)

2.2. Bioassays under controlled conditions

General conditions

Infested seeds of pea cv. Messire, collected from trials described above, were stored in paper envelopes at 4ºC. Adults of *Bp* emerging from these seeds were sexed by the presence (male) or absence (female) of a small spine on the tibia of the middle leg to (Yus Ramos, 1976). Thereafter *Bp* were separated into falcon tubes that were stored again at 4ºC were they can survive for months (Mendesil et al., 2016). Before use, adults were taken from the fridge 48 hours and placed under chamber conditions (27ºC), with water provided. Those showing greater movement were selected for the experiments.

The selected host and non-host plant species were grown under a mesh protected shelter at various planting dates with the objective to ensure a sufficient supply of clean flowers and pods at required age when needed. Plants were drip irrigated and no chemicals were applied on the plots or surroundings.

Assays were performed in a growth chamber under optimal conditions for *Bp* (27±2°C, 16L: 8D, 70% HR). Each repetition was formed by a cylindrical plastic cage (12cm diameter, 10cm depth) with a hole of 12cm^2 on the wall covered with an anti-trips mesh in order to facilitate transpiration. Wrinkled napkin paper was placed at the bottom of the cages to provide nooks where the weevils could hide. Tap water was provided by an Eppendorf sealed with cotton, twirled and stocked with adhesive tape on the wall of the cages. Flowers and pods used in each assay were placed in Eppendorf's with tap water and sealed by parafilm, (Figure 1). The positive control check used in all experiments was the susceptible pea cv. Messire; test accessions were the moderately resistant P669 (*P. sativum* ssp. *elatius*), P665 (*P. sativum* ssp. *syriacum*) and P656 (*P. fulvum*) and the non-hosts faba bean (*V. faba* cv. Brocal) and grasspea (*L. sativus* cv. Titana).

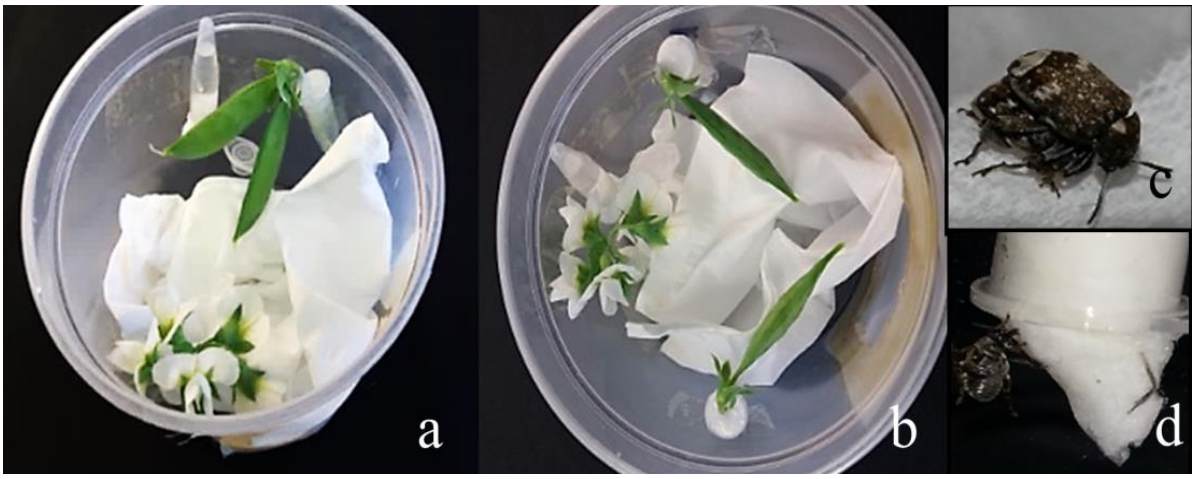

Figure 1. Plastic cages used in controlled assays to evaluate the effect of flower and pod source on B. pisorum (Bp) oviposition. a) No choice assays disposition to study the effect of host and non-host flowers on Bp oviposition capacity on pods of pea cv. Messire; b) Dual choice assay disposition to study the effect of host and non-host pods on oviposition of Bp previously feed with flowers of pea cv. Messire; c) Bp mating; d) Bp drinking water.

2.2.1. Flower source effect on *Bp* oviposition in No-Choice assay

The experimental design consisted on 10 random replications per accession (Table 2), each one consisting in a cage with five flowers of the test accession plus two pods in late flat and early swollen stage of pea cv. Messire. Four *Bp* females and two males were freed per cage and allowed to feed, mate and oviposit (Clement et al., 2002a) (Figure 1a). Flowers and pods were provided and replaced on alternated days. Cages were monitored daily to assess the days till the first oviposition and the number of eggs lied that day. Additionally adult mortality was estimated by assessing number of cages with few or many dead adults: -/+) all cages with less than 4 dead *Bp*; ++) about half of the cages with 5 to 6 dead adults; and +++) most of the cages with 5 to 6 dead adults (Table 2).

Table 2. Effect of flower genotype intake and pod offered on *Bruchus pisorum* oviposition in no-choice assays (see Fig. 1a).

		Flower source effect †			Pod source effect ‡		
Accession	**Species**	**Number of eggs laid on pods (average± SE)**	**Days till first oviposition (average± SE)**	***Bp* Mortality**	**Number of eggs laid on pods (average± SE)**	**Days till first oviposition (average± SE)**	***Bp* Mortality**
Messire	*P.s.* ssp. *sativum*	25.9 ± 4.6	5.5 ± 0.2	-/+	25.0 ± 4.2	5.0 ± 0.29	-/+
P669	*P.s.* ssp. *elatius*	19.7 ± 0.8	5.5 ± 0.2	-/+	23.4 ± 0.2	4.5 ± 0.25	-/+
P656	*P. fulvum*	14.3 ± 3.1	5.8 ± 0.5	-/+	14.0 ± 3.2	5.6 ± 0.56	-/+
P665	*P.s.* ssp. *syriacum*	18.0 ± 1.8	5.5 ± 0.2	++	10.1 ± 3.2	5.8 ± 0.53	-/+
Titana	*Lathyrus sativus*	21.1 ± 2.8	5.6 ± 0.3	-/+	1.2 ± 0.2[T]	13.4 ± 0.47 [T]	++
Brocal	*Vicia faba*	20.0 ± 2.3[α]	8.0 ± 0.4α	+++	-[§]	-[§]	+++

†: Effect of host and non-host flowers on *Bp* oviposition capacity on pea cv. Messire.

‡: Effect of host and non-host pods on oviposition of *Bp* feed with flowers of pea cv. Messire.

T: Only 6 cages showed oviposition; α: Only 4 repetitions showed oviposition; §: Some eggs were observed but never on pods; SE, standard error

2.2.2. Pod source effect on *Bp* oviposition on pods in No-Choice assay

The experimental design consisted on 15 random replicates per accession formed by a cage as described above, where 4 females and 2 males of *Bp* were freed. Each repetition consisted on five flowers of the check cv. Messire and to 2 pods of the accession to test (Table 2) in the late flat and early swollen pod stages to allow the *Bp* oviposition. Flowers and pods were provided and replaced on alternated days. P669 accession was used when there was still no presence of neoplasm formation (Np). In order to assess pod genotype effect on *Bp* oviposition, cages were monitored daily to assess the days till the first oviposition and the number of eggs lied this day. *Bp* mortality was also estimated (-/+, ++, +++) as indicated above.

2.2.3. Evaluation of *Bp* oviposition preference in Dual Choice assay

The bioassay consisted on cages as described above, containing tap water and a couple of pods, one of cv. Messire and the other from the accession to test. The pods offered for oviposition were at late flat and early swollen stages and distributed on opposite sides of the cage (Figure 1b). Two sexually mature females, previously feed on cv. Messire flowers, and 2 males were freed to allow the *Bp* oviposition as described above. In order to avoid possible stresses, also four fresh flowers of the check pea cv. Messire were provided; ten replicated per combination were performed. The number of eggs laid over each pod was assessed 24 hours after the infestation (hai).

2.3. Statistical analysis

Field data was summited to binomial probability function. No-choice data was analysed using generalized linear model (GLM) with one way analysis of variance (ANOVA) and corroborated by Poisson distribution (for count data) in order to disclose the probability of the events occurring randomly. Number of days were transformed with the logarithm of data in order to fit normal distribution and analysed by parametric ANOVA. Dual-choice data was analysed by a Student's t-test. Analyses were made by using Statistix 10 ® (Analytical Software, Tallahassee, USA).

3. RESULTS

3.1. Field Screenings

Results showed higher infestation levels at Córdoba than at Escacena (Table 1). Binomial probability function for %SI showed significant differences for both locations (Y>25; n=45; P =0.05). Accessions with the lowest %SI in both environments were P665, P669 and P656. These three accessions were selected for further bioassays under controlled conditions.

3.2. *Bruchus pisorum* bioassays under controlled conditions

3.2.1. Effect of flower source on *Bp* oviposition in no choice assay

ANOVA showed significant differences among tested accessions for the number of eggs laid (F= 2.31; df= 5; P< 0.05) although not for the number of days till first oviposition (P > 0.05). Poisson corroborates the probability of occurrence of oviposition data (*df*= 58; P=0.0001). Females fed on flowers of P665 and P656 laid significantly less eggs (Table 2). *Bp* fed on faba bean cv. Brocal showed retarded oviposition. In addition, large proportion of the adults fed on Brocal died. Mortality was moderate on adults fed on P665, but low on those fed on remaining accessions.

3.2.2. Effect of pod source on *Bp* oviposition in no choice assay

ANOVA showed significant differences among tested accessions for the number of eggs laid (F= 5.47; *df*= 4; P< 0.05) and also for the number of days till first oviposition (P <0.05). Poisson corroborates the probability of occurrence of oviposition data (*df*= 62; P=0.0001). The number of eggs laid on pods was high on cv. Messire (25 eggs/pod), being similar in P669 (*circa* 23) but significantly reduced on pods of P656 and P665 (14 and10 eggs/pod, respectively) and nil or almost negligible for the non-hosts *V. faba* cv. Brocal and *L. sativus* cv. Titana. Number of days required for first oviposition was similar among *Pisum* accessions (range 4.5 – 5.8 days), but highly retarded on pods of *L. sativus* cv. Titana (13.4 days). No eggs were laid on pods of *V. faba* but on any place in those cages, either on the parafilm or the cage walls (Figure 2). Only in one repetition 1 egg was observed on a *V. faba* pod, what was not included in the analyses. In addition, large proportion of the adults offered pods of *V. faba* died. Mortality was higher on the cages with *L. sativus* pods in comparison with those fed on remaining accessions.

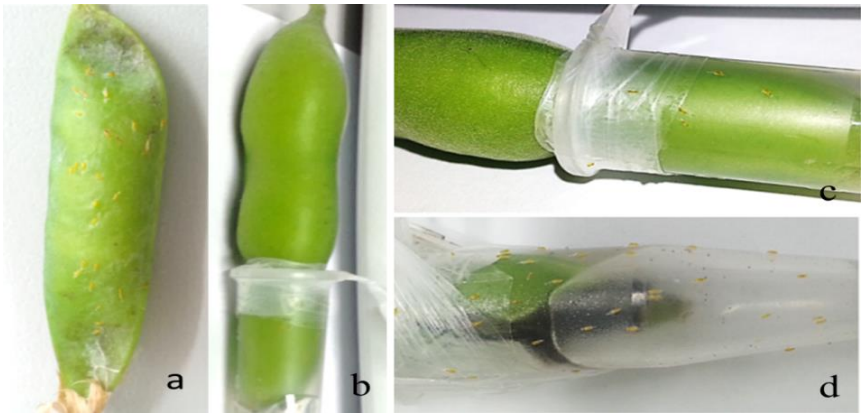

Figure 2. Effect of pod genotype on oviposition of *B. pisorum* females previously feed with pollen of pea cv. Messire. a) Messire pod with abundant eggs laid; b) V. *faba* pod without eggs laid; c) and d) details of b) showing eggs lied elsewhere (on top of the parafilm and Eppendorf tube) but not on pod.

In order to corroborate the high morality observed in cages with *V. faba* pods, 6 additional repetitions were performed under the same conditions described on 2.2.2. In all cages *Bp* died before the oviposition (data not shown).

3.2.3. Evaluation of *Bp* oviposition preference in Dual Choice assay

In dual choice assays Messire was generally preferred for oviposition. Accessions confronted with Messire showed significant reduced oviposition in cages containing P665 (T= 3.73; *df=9*; P=0.005), *V. faba* (T= 5.65; *df*=9; P=0.0003), *L. sativus* (T= 9.54; *df=9*; P=0.0001) or P656 (T= 3.81; *df*=9; P=0.004). No eggs were laid on *V. faba* pods. Conversely, P669 was preferred for oviposition than Messire (T= -3.91; *df*=9; P=0.003) (Figure 3). In addition, the total amount of eggs laid on P656-Messire couple was significantly higher than for the

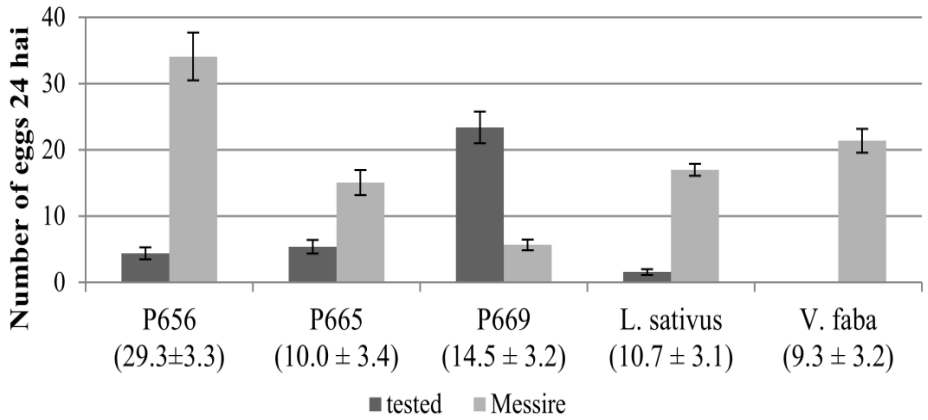

Figure 3. Oviposition of *B. pisorum* in dual choice assays. Columns show the mean of number of eggs lied over pods of tested accessions versus pea cv. Messire (control). Bars showed the standard error; numbers over tested accessions indicate the total amount of eggs lied per couple with standard error.

remaining couples (Figure 3).

4. DISCUSSION

Resistance to pea weevil is a major priority for pea breeding. Field screenings corroborated the environmental effect on seed infestation and highlighted the higher resistance of P656, P665, P669 accessions in both environments evaluated in agreement with (Aznar-Fernández et al., 2017). Bioassays reported here corroborated that both the flower and the pod source influence on *Bp* oviposition. The importance of pollen and nectar consumption in *Bp* oviposition is largely known (Clement, 1992, Wäckers et al., 2007). In our study, the source of pollen influenced the number of eggs laid. In addition *V. faba* caused a high *Bp* mortality and retarded oviposition, which corroborate that pollen and nectar directly affects the sexual maturity of *Bp* females (Pesho and Van Hounten, 1982). However, results showed that females could also sexually mature by feeding from non-host species flowers such as *L. sativus*, what is in agreement with (Barry and O'Keeffe, 1984) who reported that sexual maturation of *Bp* females were ascribed to the amounts of pollen ingested rather than differences in nutritional quality of different pollens. Moreover this behaviour could prolong *Bp* life-span (Pajni, 1981) and benefit their dispersal by providing a source of energy to sustain flight after hibernation (Clement, 1992). Our study shows that the source of flowers offered might reduce (the case of various *Pisum* accessions) or even retards oviposition and cause adult mortality (the case of *V. faba*). This could be due to the primary metabolites, which are important feeding stimulants for Coleoptera, and might be different between species and genotypes (Wäckers et al., 2005). Further studies are needed to discern if the retarded and reduced oviposition of females feed on *V. faba* flowers are due just to amount of pollen and/or nectar eaten or to anti-nutritional effects. The mortality of adults fed with *V. faba* and P665 flowers might suggest some anti nutritional effect (Table 2). Interestingly accessions P665, P656, P669 and Brocal showed flower pigmentation known to be associated with condensed tannins (Wang et al., 1998). Tannins are widely recognized as plant defence against herbivore insects (Barbehenn and Constabel, 2011) and could act as deterrents for feeding under natural conditions.

On the other hand, there was a strong effect of pod offered on preference for oviposition with significant effects on the number of eggs laid, and days till first oviposition on non-host species. This suggests the crucial role during weevil oviposition of plant genotype. Oviposition was particularly affected on *V. faba* and *L. sativus* with a marked reduction in the

number of eggs laid and a delayed start of oviposition on *L. sativus*. There was also a significant reduction of number of eggs laid on *P. sativum* ssp. *syriacum* (P665) and *P. fulvum* (P656). As described before, P665 accession showed purple pigmentation also in pods. Antixenosis on *P. fulvum* pods has been previously described (Hardie and Clement, 2001), as well as antibiosis (Clement et al., 2002b). *Bp* oviposition repellence or deterrence over pods might be to structural defence mechanism such as the odour, touch, thickness, colour, trichomes (Edwards and Singh, 2006b, Mendesil et al., 2016) wax layer (Chang et al., 2006) and also for secondary metabolites such as volatiles (Bruce et al., 2011, Ceballos et al., 2015) or plant defence responses to *Bp* presence (Bennett and Wallsgrove, 1994). The length of pea pods could also interfere in *Bp* preference for oviposition (Hardie and Clement, 2001), however in the late flat and early swollen stage of pods from our bioassays pea accession displayed similar lengths.

Our results show strong deterrence against non-host *V. faba* cv. Brocal pods, forcing females to oviposit elsewhere but not over *V. faba* pod (Figure 2). Data of mortality, displayed by both non-hot species suggest that such deterrence might influence on *Bp* lifespan inside cages.

P. sativum ssp. *elatius* accession P669 showed reduced consistent seed infestation in field screenings under multiple environments (section 2.1 and (Aznar-Fernández et al., 2017)). This might be due to neoplasm (*Np*) formation often observed on this accession, although the effect has been not quantified. *Np* formation has been reported to reduce the efficiency of *Bp* larval penetration ((Doss et al., 1995b) being the reduction in oviposition associated with the level of *Np* formation (Mendesil et al., 2016).Our experiments show that young pods of P669 pods are not deterrent, suggesting that the reduction on infestation under field conditions might indeed be due to effects of *Np*. However, we used young pods, before *Np* were formed, and therefore cannot discern if the reduced infestation of P669 is due *Np* reducing oviposition and/or hampering successful larva occlusion and penetration on pods. In dual choice assays no eggs were laid on pods of *V: faba*. This egg-lying deterrence might be useful for *Bp* management in pea intercropped with this deterrent accession, what deserves further investigations. Accessions P665, P656 and *L. sativus* showed no preference for oviposition in front Messire; however P656-Messire couple displayed the highest amount of eggs, which demonstrate no deterrence on *Bp* egg lying. As described before, pea pods thickness, wax, trichomes, etc. could play a major role on *Bp* oviposition preference; meanwhile non-host plants have shown several metabolites and pheromones acting as oviposition-deterrence on non-target insects(Cook et al., 2007)

Results of this study suggest the use P665 and *V. faba* as promising combinations in intercropping (Finch and Collier, 2012).; being both push-pull strategies which modify the pest behaviour in order to reduce *Bp* pressure on the crop (Cook et al., 2007). In addition data revealed the appropriateness of *L. sativus* pollen for *Bp* oviposition over pea pods, but not in *Lathyrus*; suggesting the inappropriateness of sown these two crops together. Albeit, studies under filed conditions should be carried out in order to could state right conclusions.

5. ACNOWLEDGMENTS

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CHAPTER 3

Identification of quantitative trait loci (QTL) controlling Seed infestation and larval development of pea weevil (*Bruchus pisorum*) in a high-density integrated DArTseq SNP-based genetic map of pea

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In progress

TITLE:

Identification of quantitative trait loci (QTL) controlling resistance to pea weevil (*Bruchus pisorum*) in a high-density integrated DArTseq SNP-based genetic map of pea

ABSTRACT:

Pea weevil (*Bruchus pisorum*) is a damaging insect pest affecting pea (*Pisum sativum*) production worldwide to which no resistant cultivars are available. Some levels of incomplete resistance have been identified in *Pisum* germplasm, including *P. sativum* ssp. *syriacum* P665 accession. To decipher the genetic control underlying this resistance, a recombinant inbred line (RIL $F_{8:9}$) population from a cross between the resistant P665 and the susceptible pea cv. Messire was genotyped using Diversity Arrays Technology and screened under field conditions for seed infestation and larval development along 5 environments. A newly integrated genetic linkage map was generated with a subset of 10,399 markers, assembled into seven linkage groups, equivalent to the number of haploid pea chromosomes. An accumulated distance of 2,503 cM was covered with an average density of 4.11 markers cM^{-1} and an average distance between adjacent markers of 1.56 cM. The linkage map allowed the identification of three QTLs associated to reduced seed infestation along LGs I, II and IV, with and individual contribution to the total phenotypic variance ranging between 14.8 and 24%. In addition, one QTL for reduced larval development was identified in LGIV, explaining approximately 16 % of the phenotypic variation. Expression of these QTLs varied with the environment, being particularly interesting for its stability QTL BpSI.III that was detected in 5 environments. This is the first mapping analysis for pea weevil resistance in a high-saturated pea genetic map originates from in silicoDArT-derived markers, which has allowed the identification of potential

candidate genes co-located with QTLs for marker-assisted selection, providing an opportunity for breeders to generate effective and sustainable strategies for weevil control.

KEYWORDS: *Bruchus pisorum*, pea, seed infestation, larval development, QTL, DArTSeq

1. INTRODUCTION

Pea (*Pisum sativum* L.) is the second most cultivated temperate grain legume in the world and the first in Europe (FAOSTAT, 2016). Its use extends to human consumption and animal feed (Smýkal et al., 2012). As most crops, pea can be damaged by a range of pests and diseases (Rubiales et al., 2015b). One of the most intractable and harmful ones worldwide is pea weevil (*Bruchus pisorum* L., Coleoptera: Bruchidae, *Bp*) causing seed yield losses of up to 50% (Clement et al., 2002b, Keneni et al., 2011). *Bp* is a strict monophagus insect (Tibor and Szentesi, 2003) whose adults *Bp* adults remain dormant in winter and reactivate when temperatures increase in coincidence with pea blooming. Females lie eggs on young pea pods and emerging larvae penetrates through the pods into the seeds, eating the cotyledon and finally moulting inside the seeds (Hardie and Clement, 2001). *Bp* control by insecticides is complicated by the fact that most of the insect life cycle is completed inside the seeds. Consequently, for the effectiveness of chemical treatments a constant monitoring on field is required to adjust the spray timing with *Bp* oviposition (Horne and Bailey, 1991a). In addition postharvest fumigations in storehouses might be needed to reduce the emergence of *Bp* that are hibernating or developing inside the seeds (Clement et al., 2009). Because of the high ecologic and economic cost of chemical control, other strategies such as biological control with parasitoids (Barry and O'Keeffe, 1987, Huis et al., 1990), early sowing, removal of crop residues (Teshome et al., 2015) or intercropping (Teshome et al., 2016) have been attempted. Unfortunately none of them provided sufficient level of control so far. This reinforce the need to invest on the development of resistant cultivars for a more economic, ecologic and efficient *Bp* management.

The lack of *Bp* resistance in pea cultivars forced the search for resistance in germplasm collections, including landraces and wild relative species. Resistance has been identified in accessions of *P. sativum* and wild relatives (Teshome et al., 2015). Out of these, resistance of *P. sativum* ssp. *syriacum* accession P665 showed highest and more stable levels of *Bp* resistance

in field screenings (Aznar-Fernández et al., 2017). This resistance was confirmed under controlled conditions, showing to be a combination of antixenosis and antibiosis resulting in reduced seed infestation and retarded larval development (Aznar-Fernández and Rubiales, 2018 a). This accession was previously identified as resistant to a number of other stresses such as ascochyta blight (*Dydimella pinodes*) (Fondevilla et al., 2005), broomrape (*Orobanche crenata*) (Rubiales et al., 2005b), pea aphid (*Acyrtosiphon pisum*) (Aznar- Fernández and Rubiales, 2018 b), and drought (Iglesias-García et al., 2015), and therefore it was early introduced in the crossing program and a number of breeding populations were generated that are extensively used at the breeding program. In addition, a RIL population was generated from the cross with pea cv. Messire that was used in previous genetic studies (Fondevilla et al., 2012). The RIL is nowadays in an advanced generation ($F_{8:9}$), avoiding the possible resulting distortion and is considered a valuable resource to unravel genetics of *Bp* resistance upon new high throughput marker deep genotyping.

The use of molecular markers linked to resistance genes seems to be an affordable and competent way to reduce efforts, time of evaluations, economic costs and do more efficient and effective the traditional plant breeding programs developed for pest control (Rubiales et al., 2015b). In order to achieve reliable information regarding the genomic regions involved in resistance, the precision in trait scoring and the availability of high density genetic maps are crucial. In this scenario, the Diversity Arrays Technology (DArT) in combination with next-generation sequencing platforms (Kilian et al., 2012, Raman et al., 2014) known as DArTseq™, provides a good choice as a high throughput marker genotyping platform that can develop a relatively large number of polymorphic markers to build dense genetic maps with low-cost investments (Barilli et al., 2018b).

Therefore, the objectives of the present study were the development of the first integrated high-density DArTseq based genetic linkage map of the *P. sativum* ssp. *syryacum* (P665) x *P. sativum* ssp. *sativum* (cv. Messire) RIL $F_{8:9}$ population, as well as the identification of regions in the pea genome controlling *Bp* seed infestation and larval development.

2. MATERIAL & METHODS

2.1. Plant material

The population used in the study was composed of 108 recombinant inbred lines (RILs) ($F_{8:9}$) derived by a single seed method from a cross between the *Bp* resistant *Pisum sativum* ssp*. syriacum* accession P665 and the susceptible *P. sativum* ssp. *sativum* cv. Messire.

Response to *Bp* infestation of the RIL population together with their parental lines was studied under field conditions in a total of five environments (combination of location and year). These were Córdoba (CORD) during growing seasons 2014-15 and 2015-16, Escacena del Campo (ESC) seasons 2013-14 and 2014-15 and Espiel (ESP) in growing season 2015-16. Seed infestation (SI) was assessed in all environments, whereas larval development (LD) was assessed on three environments only, as indicated in Table 1 where climatic data for each environment are also provided.

Table 1: Environmental conditions at the different experimental sites and years.

Environment	Location	Season	Av. Temp (ºC)	Av. Humidity (%)	Accu. Rainfall (mm)	Accu. Rad (W/m2)
ESC1	**Escacena del Campo**	2013-2014	15.4	72.27	376.4	16.6
ESC2	**Escacena del Campo**	2014-2015	15.2	66.1	130.0	17.7
CORD1	**Cordoba**	2014-2015	15.0	66.8	164.1	17.4
CORD2	**Cordoba**	2015-2016	15.0	69.9	354.2	16.1
ESP	**Espiel**	2015-2016	12.6	71.5	251.6	15.6

The parameters (Av. T: average temperature; Av humidity: average humidity; Accu. rainfall: accumulated rainfall; Accu.Rad: accumulated radiation) are given for the crop season (from sowing till harvest date).

Field assays were designed as a randomize block design with 4 repetitions. Experimental unit consisted in a 50 cm long row where 7-10 seeds for a line were sown. Rows were separated 50 cm between each other. In order to ensure a homogenous germination, seeds were previously scarified. No pesticide or herbicide was applied on trials and only mechanical weeding was done. In order to ensure a high a uniform infestation, 100 *Bp* adults (obtained from infested seeds of previous seasons) were freed in each field by middle March, when natural infestation starts (Aznar-Fernández et al., 2017).

Plants were manually harvested at maturity, threshed and seeds stored at 4ºC. The evaluation of SI and LD was done by opening through the cotyledons of 100 seeds randomly

taken from each replication (Aznar-Fernández et al., 2017). SI was calculated as the percentage of seeds showing infestation at any stage of development. Stage of development of the larva (LS) in each seed was assessed was with the help of a visual scale (Clement et al., 2002b) slightly modified where: LS1 = first instar-larval penetration, 0–5% cotyledon eaten; LS2 = 6–25% cotyledon eaten; LS3 = 26–60% cotyledon eaten, second to fourth instar; LS4 = extensive damage, pre-pupa; LS5 = adult (Figure 1). A larval development index (LD) was calculated as follow: LD$= \frac{\sum \text{LS x Ni}}{5 \text{ x Nt}} x100$; where LS is the larval stage, Ni is the number of seeds at each LS and Nt is the amount of Nt for each treatment, 5 showed the number of the total stages from the visual scale used in this study.

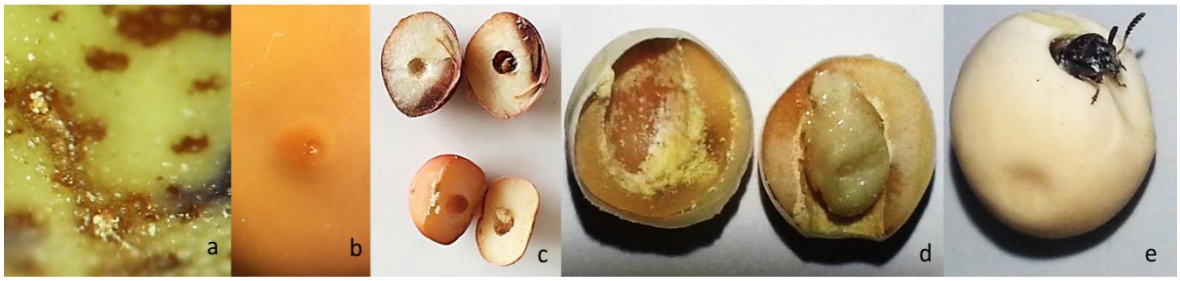

Figure 1. Seed infestation and larval stages (LS) of *Bruchus pisorum* (*Bp*) in parental lines, (a-b) holes of *Bp* larvae penetration (a) resistant parental line P665; (b) susceptible parental line cv. Messire; (c-d) opening of the seeds thorough the cotyledon to check the presence or absence of *Bp* larva inside seeds and LS; (c) LS2; (d) LS4; (e) *Bp* adult emergence from Messire, LS5 .

Analyses for phenotypic traits were made by using Statistix 10 ® (Analytical Software, Tallahassee, USA). The percentage data for SI was Ln transformed before subjecting to analysis of variance (ANOVA). Data for LD was analysed using generalized linear model (GLM) with one way analysis of variance (ANOVA). Pearson's linear correlation was calculated to study the possible relationship between the parameters evaluated.

2.2. Genetic map development

2.2.1. DNA extraction and quantification

Seedlings of all lines were grown under controlled conditions at CSIC Córdoba. Around 1 g of young leaf tissue from the 3rd-4th node of each plant was excised, immediately frozen in liquid nitrogen and stored at -80ºC. Genomic DNA was isolated from fresh and young leaves of plants using a modified cetyltrimethylammonium bromide (CTAB)/chloroform/isoamylalcohol method (Doyle and Doyle, 1987). DNA quantification was done by agarose gel electrophoresis (0.8 %), and it was adjusted to50 ng/µl for DArT and SNP genotyping.

2.2.2. Genotyping by DArTseq technology

A high-throughput genotyping method using the DArT-Seq™ technology at Diversity Arrays Technology Pty Ltd (Canberra, Australia) was employed to genotype the RIL population. Essentially, DArT-Seq™ technology relies on a complexity reduction method to enrich genomic representations with single copy sequences and subsequently perform next-generation sequencing using HiSeq2000 (Illumina, USA). DArT-Seq detects both SNPs and presence–absence sequence variants, collectively referred to as DArT-Seq markers (Raman et al., 2014). DNA samples are processed in digestion/ligation reactions Kilian et al., (2012), but replacing a single *PstI*-compatible adapter with two different adapters corresponding to two different restriction enzymes (RE) overhangs. The *PstI*-compatible adapter was designed to include Illumina flowcell attachment sequence, sequencing primer sequence and staggered, varying length barcode region. The reverse adapter contained the flowcell attachment region and *Mse*I-compatible overhang sequence. Only "mixed fragments" (*PstI*–*MseI*) were effectively amplified in 30 rounds of PCR using the following reaction conditions: 1 min at 94 °C for initial denaturation; 30 cycles each consisting of 20 s at 94 °C for denaturation, 30 s at 58 °C for annealing and 45 s at 72 °C for extension; and finally a 7 min extension step at 72 °C. After PCR, equimolar amounts of amplification products from each sample of the 96-well microtiter plate were bulked and applied to c-Bot (Illumina) bridge PCR followed by sequencing on Illumina Hiseq2000. The sequencing (single read) was run for 77 cycles. Sequences generated from each lane were processed using proprietary DArT analytical pipelines. In the primary pipeline, the FASTQ files were first processed to filter poor-quality sequences, applying more stringent selection criteria to the barcode region compared to the rest of the sequence. Thus, the assignments of the sequences to specific samples carried in the "barcode split" step are more consistent. Approximately 2,500,000 (approximately 7%) sequences per barcode/sample are used in marker calling. Finally, identical sequences are collapsed into "fastqcall files". These files were used in the secondary pipeline for DArT P/L's proprietary SNP and SilicoDArT (Presence/Absence Markers in genomic representations) (present = 1 vs. absent = 0) calling algorithms (DArTsoft14). The analytical pipeline processed the sequence data.

The parameters used for quality control at the time of selecting high-quality SilicoDArT and derived SNPs markers (Kilian et al., 2012) for genetic mapping were: the reproducibility of 100%; the overall call rate (percentage of valid scores in all possible scores for a marker) over 95%; the polymorphic information content (PIC) between 0.3 and 0.5 and the Q value (the

logarithm of the minimum false discovery rate at which the test may be called significant) above 2.5.

2.2.3. Linkage map and QTL mapping

The scores of all polymorphic DArTseq and SNP markers were converted into genotype codes ("A", "B") according to the scores of the parents. Linkage groups (LG) were obtained using the software JoinMap version 4.1 (Van Ooijen, 2006). The maximum likelihood mapping algorithm, which was optimised for constructing dense genetic maps using this software (Jansen et al., 2001), was first used for grouping all of the polymorphic markers. Then, the method of regression mapping (Haley and Knott, 1992) was used for map construction with approximately 1,000 markers with appropriate genetic distance and the marker position, and the order of markers for three rounds to merge the tightly adjacent markers into bins. The markers in adjacent loci with genetic distance below 0.2 cM were classified into a bin during the first two rounds of mapping. Moreover, one marker with sequence information and with the least missing genotype from each bin was chosen as a "bin representative" for the next round of genetic mapping. In a third round of mapping, the makers in adjacent loci pairs with genetic distances below 0.1 cM were classified into a bin to avoid incorrect classification when the markers were decreased in the map. The Kosambi mapping function (Kosambi, 1943) was used to convert recombination frequencies into map distances, and only "Map 1" was used for further analysis. The linkage groups maps of each chromosome were drawn and aligned using MapChart v2.3 (Voorrips, 2002). The threshold for the goodness of fit was set to ≤ 5.0, with a recombination frequency of <0.4. Linkage groups were separated using a LOD score ≥ 3.0. Markers with a mean Chi Squared value of recombination frequency > 4.0 were discarded, according with Barilli et al. (2018). DArT markers were named with the numbers corresponding to unique clone ID following Kilian et al., (2012).

SNP markers previously mapped in this RIL were used as ("anchor marker") to find the correspondence between this new map with previously published versions (Fondevilla et al., 2012, Carrillo et al., 2014) and to assign the *P. sativum* ssp. *syriacum* x *P. sativum* linkage groups to pea chromosomes. For the same purpose, the sequences from DArT-seq-derived markers were compared with *Cicer arietinum* and *Medicago truncatula* genomic backbones by using Phytozome v.12 (https://phytozome.jgi.doe.gov/pz/portal.html) to perform a synteny analysis using three parameters recently defined by (Salse et al., 2009). These parameters increase the stringency and significance of BLAST sequence alignment by parsing BLASTX results and rebuilding HSPs (High Scoring Pairs) or pairwise sequence alignments to identify

accurate paralogous and orthologous relationships. This analysis allowed searching for sequence similarity-based homology between legume species providing an alternative approach to finding correspondence between linkage groups.

QTL analysis for *Bp* resistance was conducted using composite interval mapping (CIM) and multiple interval mapping (MIM) in MapQTL 6.0 package (Van Ooijen, 2006). Markers to be used as cofactors for CIM were selected by forward–backward stepwise regression. Significance thresholds of log of odds (LOD) corresponding to a genome-wide confidence level of $P < 0.05$ were determined for each trait using the permutation test of MapQTL 6.0 with 1000 iterations, according with Barilli et al., (2010). Skewness and Kurtosis coefficients were calculated following Lynch and Walsh, (1997) procedure. The coefficient of determination (R^2) for the marker most tightly linked to a QTL was used to estimate the proportion of the total phenotypic variation explained by the QTL.

3. RESULTS

3.1. SI and LD assessment

Seed infestation under field conditions showed contrasting values, being parental line P665 significantly more resistant than parental line Messire ($P < 0.05$) in all environments, confirming previous findings (Aznar-Fernández et al., 2017). Analysis of variance of each trial revealed highly significant genotypic effects for SI resistance criteria among the RIL families ($P < 0.05$) (Figure 2). Distribution of SI in the RIL population in each environment followed a normal distribution according to Lilliefors (Kolmogorov-Smirnov) normality test ($P > 0.05$). The coefficient of Skewness in field conditions was of 0.27, 0.15, 1.04, 1.48 and 1.79 in ESC1, CORD1, ESC2, CORD2 and ESP, respectively, showing skewness towards reduced SI. Average values for SI measured on the whole RIL population showed significant differences between locations and years, being higher in ESC1 (38.2%) and lower in ESP (22.92%) (Table 2).

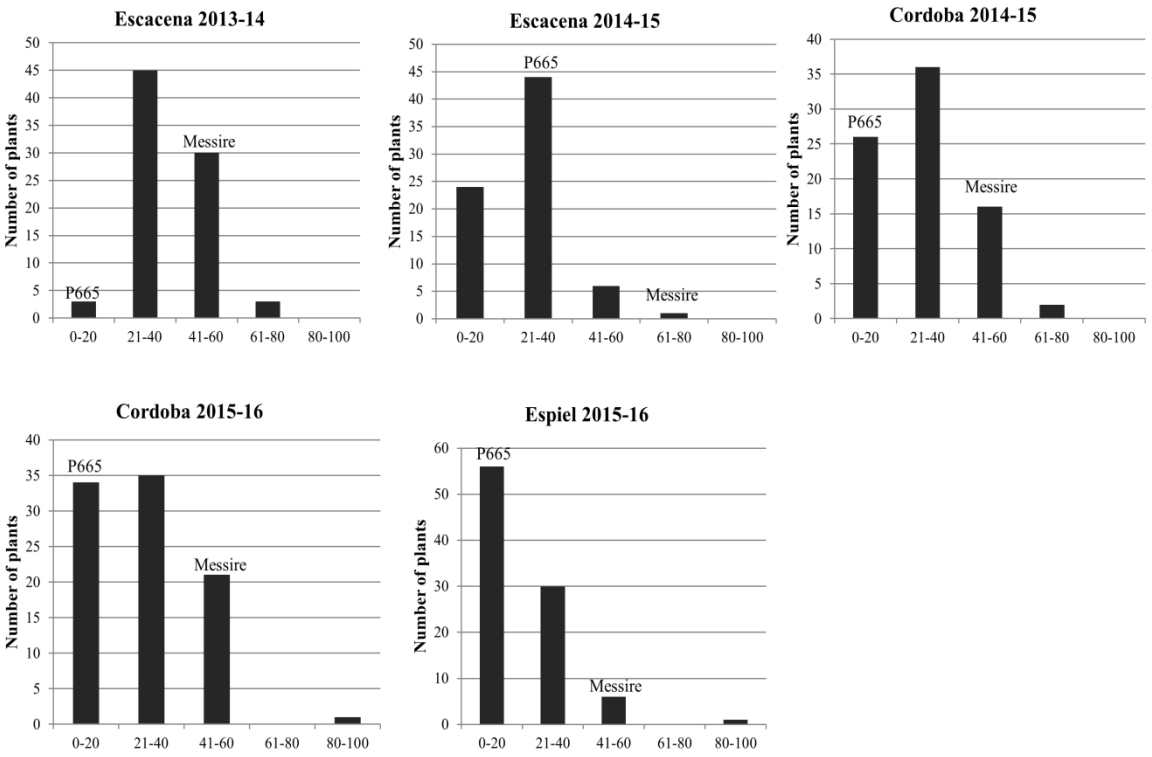

Figure 2. Frecuency distribution among RIL population from (P665 x Messire) cross in response to *Bruchus pisrum* seed infestation (%) under field conditions in five environments.

Table 2: Response to *Bp* infestation: mean values of seed infection (SI) and larval development (LD) measured on the RIL population at the different environments.

Environment	SI (%) Mean ± SE	LD Mean ± SE
ESC1	38.20 ± 1.3***	nd
ESC2	27.10 ± 1.3***	nd
CORD1	28.16 ± 1.7***	70.9 ± 2.2***
CORD2	31.33 ± 2.5***	55.4 ± 1.4***
ESP	22.92 ± 2.2***	62.8± 1.7***

***Significant differences of each environment at P = 0.001; **significant at P = 0.01; *significant at P = 0.5; ns = not significant; SE: standard error of the mean; nd: not determined

Parental lines showed also contrasting LD values in all environments evaluated, being < 20 for P665 and > 68 for cv. Messire (Figure 3). LD did not follow a normal distribution

(Lilliefors (Kolmogorov-Smirnov) normality test; $P < 0.05$). One way ANOVA for LD showed significant difference in the RIL population in all environments ($P < 0.05$) (Table 2). The coefficient of Skewness for LD was of -1.08, -0.89 and -1.77 in CORD1, CORD2 and ESP, respectively, showing skewness towards high LD. In fact, several transgressive RIL families showed higher LD values than the susceptible parental line, Messire (Figure 3).

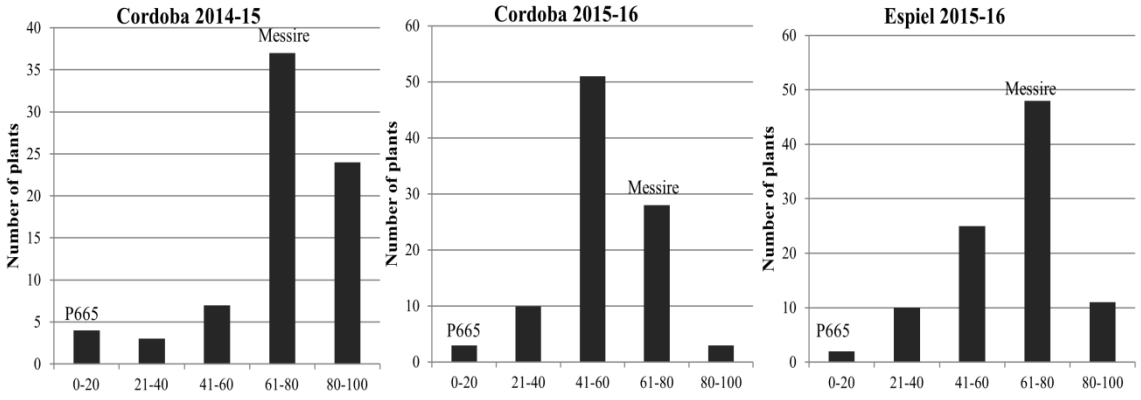

Figure 3. Frecuency distribution among RIL population from (P665 x Messire) cross in response to *Bruchus pisrum* larval development (LD) under field conditions in three environements.

Pearson's linear correlation coefficients (Table 3) were generally significant between SI values evaluated in the field within years and locations, as well as between LD values.

Table 3: Pearson's linear correlation coefficient between seed infestation (SI) and larval development (LD) at the various environments.

	SI ESC1	SI ESC2	SI CORD1	SI CORD2	SI ESP	LD CORD1	LD CORD2
SI ESC2	0.52***						
SI CORD1	0.65***	0.43***					
SI CORD2	0.56***	0.61***	0.60***				
SI ESP	0.44***	0.55***	0.50***	0.40***			
LD CORD1	-	-	0.60***	0.30**	0.32**		
LD CORD2	-	-	0.44***	0.61***	0.42***	0.38***	
LD ESP	-	-	0.66***	0.41***	0.48***	0.63***	0.62***

***Significant at P = 0.001; **significant at P = 0.01; *significant at P = 0.5; ns = not significant.

3.2 Genotyping and Linkage Mapping

A total of 12,012 high-quality SilicoDArT and 14,880 SNPs markers were identified, for a total of 26,892 DArT-derived markers. Of these, a set of 10,287 markers (38.2%) were selected for mapping after quality filtering. The mapping dataset was complemented with 112 SNP markers previously mapped in this RIL (Carrillo et al., 2014). All non-DArT derived markers mapped in the expected LGs, accordingly with previous publications (Deulvot et al., 2010, Fondevilla et al., 2012, Carrillo et al., 2014). Markers were distributed across 7 LGs using LOD thresholds ranging from 3 to 10 and a recombination frequency (r) threshold < 0.4 (JoinMap vs. 4). Each assigned group included at least ten markers common to other published *P. sativum* genetic maps.

The newly constructed integrated genetic linkage map of *P. sativum* ssp. *syriacum* x *P. sativum* ssp. *sativum* covered a total length of 2,503.6 cM, with an average density of 4.11 markers cM^{-1} and an average adjacent-marker gap distance of 1.6 cM (Table 4). The total number of mapped loci per linkage group ranged from 1087 on LGVII to 2041 on the LGI, and the average was of 1,5 loci LG^{-1}. The longest individual linkage group map was for the LGI (448.42 cM), the shortest was for the LGVI (286.38 cM) (Table 4), and the average LG length was 357.66 cM. The density of markers in the individual linkage groups ranged from 3.26 markers cM^{-1} in the LGVII to 4.77 markers cM^{-1} in the LGVI. Map distances between two consecutive markers varied from 0 to 7.86 cM, while the gap average between markers varied from 1.49 cM in the LGI and 1.84 cM in the LGVII (Table 4).

Table 4: Map features of P665 x Messire linkage map.

Linkage group	DArT-seq markers	Other markers	Unique position	Distance (cM)	Average gap distance (cM)	Larger gap (cM)	Trimmed sequences		Corresponding pea chromosome
							Medicago truncatula	*Cicer arietinum*	
LGI	2,041	27	303	448.42	1.49	6.32	100	107	3
LGII	1,601	18	262	394.78	1.51	7.37	32	47	7
LGIII	1,491	10	252	396.88	1.58	7.37	31	23	4
LGIV	1,446	14	209	313.71	1.50	7.40	28	34	2
LGV	1,254	17	222	330.60	1.49	6.31	19	21	6
LGVI	1,367	14	184	286.38	1.56	7.86	16	28	5
LGVII	1,087	12	181	332.88	1.84	6.38	24	24	1
Total	*10,287*	*112*	*1,613*	*2,503.65*	*1.56*	*7.01*	*250*	*284*	

Two hundred and 284 sequences from the DArTseq-derived markers were respectively BLASted with *M. truncatula* and *C. arietinum* genomes, what together with the used of 112 previously mapped SNPs markers, allowed to define the correspondence between LGs from *P. sativum* ssp. *syriacum* x *P. sativum* ssp. *sativum* cross and their pea chromosome assignment, as follows: 27 SNP markers as well as 207 DArTseq-derived markers linked the LGI to the *P. sativum* LG3; 18 SNP markers, as well as 79 DArTseq-derived markers linked LGII to LG7; 10 previously reported markers, as well as 54 DArTseq-derived markers linked LGIII to LG4; 14 SNPs and 62 DArTseq-derived markers related LGIV to LG2; 17 previously reported SNP markers and 40 DArTseq-derived markers related the LGV to the *P. sativum* LG6; 14 SNPs and 44 DArTseq-derived markers linked the LGVI to the *P. sativum* LG5; finally, 12 previously mapped SNP markers and 48 DArTseq-derived markers showed that the LGVII corresponds with the LG1 (Table 4).

3.3. QTL's analysis

Quantitative trait loci analysis with composite interval mapping (CIM) and multiple interval mapping (MIM) methods revealed genomic regions involved resistance to *Bp* assessed as reduction of SI and LD in LGs I, II and IV.

QTL where not significant at all environments studied, but showed a similar tendency although with low LOD values (Table 5). Significant QTLs explained from 12.6 to 20.64% of weevil SI variation depending on the environment.

QTL *BpSI.I* was significant at ESC1 and ESC2 environments; with LOD score of 3.00 and 4.17 and explained 14.8 and 24.3% of SI variation, respectively. Meanwhile there was no peak present in the other environments. *BpSI.I* peak was localized for both environments scored at 112.6 cM from the beginning of the LGI, between the DArT markers 35529271 and 3552969 (Table 5, Figure 4). The distance to the left and to the right flanking markers ranged between 1.05 – 1.05 cM, respectively.

The second QTL, *BpSI.II*, showed a LOD score of 4.32 and explained 19.2% of the phenotypic variation under ESP environmental conditions (Table 5, Figure 4). *BpSI.II* was localized at 161.5 cM from the beginning of LGII, between DArT markers 3551915 and 3541563. The distance to the left and to the right flanking markers was of 1.05 – 2.1 cM, respectively. Interestingly, similar peaks were found in the same region (185.2 cM) at ESC1 and CORD1 environments but with LOD values under the threshold (LOD 2.27 and 2.57 respectively).

Table 5: Position and effects of quantitative trait loci (QTL) for plant resistance to *B. pisorum* based on percentage of seed infection (SI) and larval development (LD) measured in field over five different environments using composite interval mapping (CIM) and multiple interval mapping (MIM) by MapQTL 6.0 in the P665 x Messire RIL population.

QTL[a]	LG[b]	Trait[c]	Peak[d]	Flanking markers		LOD[e]	Add[f]	R^{2g}
BpSI.I	I	SI ESC1	112.6	35529271	3552969	3.00	-4.42	14.8
		SI ESC2	112.6	35529271	3552969	4.17	-0.24	24.3
BpSI.II	II	SI ESP	169.5	3551915	3541563	4.32	-0.48	19.2
		SI ESC1*	185.26	3552982	3549468	2.27	-0.13	7.3
		SI CORD1*	185.26	3552982	3549468	2.57	-0.27	9.1
BpSI.III	IV	SI ESC2	164.22	3550927	3552154	3.72	-0.22	22.0
		SI ESP	183.20	3551009	3543841	4.26	-0.46	19.0
		SI CORD2	172.64	3548130	3545955	3.94	-0.42	18.4
		SI ESC1*	*162.12*	*3542500*	*3542038*	*2.28*	*-0.16*	*12.7*
		SI CORD1*	*175.8*	*3545955*	*3542026*	*2.01*	*-0.30*	*11.5*
BpLD.I	IV	LD ESP	175.8	3545955	3542026	3.51	0.12	16.1
		LD CORD1*	*176.8*	*3543378*	*3550725*	*2.28*	*0.03*	*7.5*

[a]QTL that extend across single one-log support confidence intervals were assigned the same symbol
[b] *LG* linkage group
[c]SI CORD1, SI CORD2, SI ESC1, SIESC2 and SI ESP: seed infection (%) under field conditions measured at Córdoba (Spain) during growing seasons 2014/15, 2015/16, at Escacena del Campo (Spain) during seasons 2013/14 and 2014/15 and at Espiel (Spain) during season 2015/16, respectively.
[d] *Peak* QTL position (cM)
[e] *LOD* the peak LOD score
[f] *Add* the additive effect
[g] R^2 proportion of phenotypic variance explained by the respective QTL (%)
[*] QTL following the same tendency but with no significant LOD values.

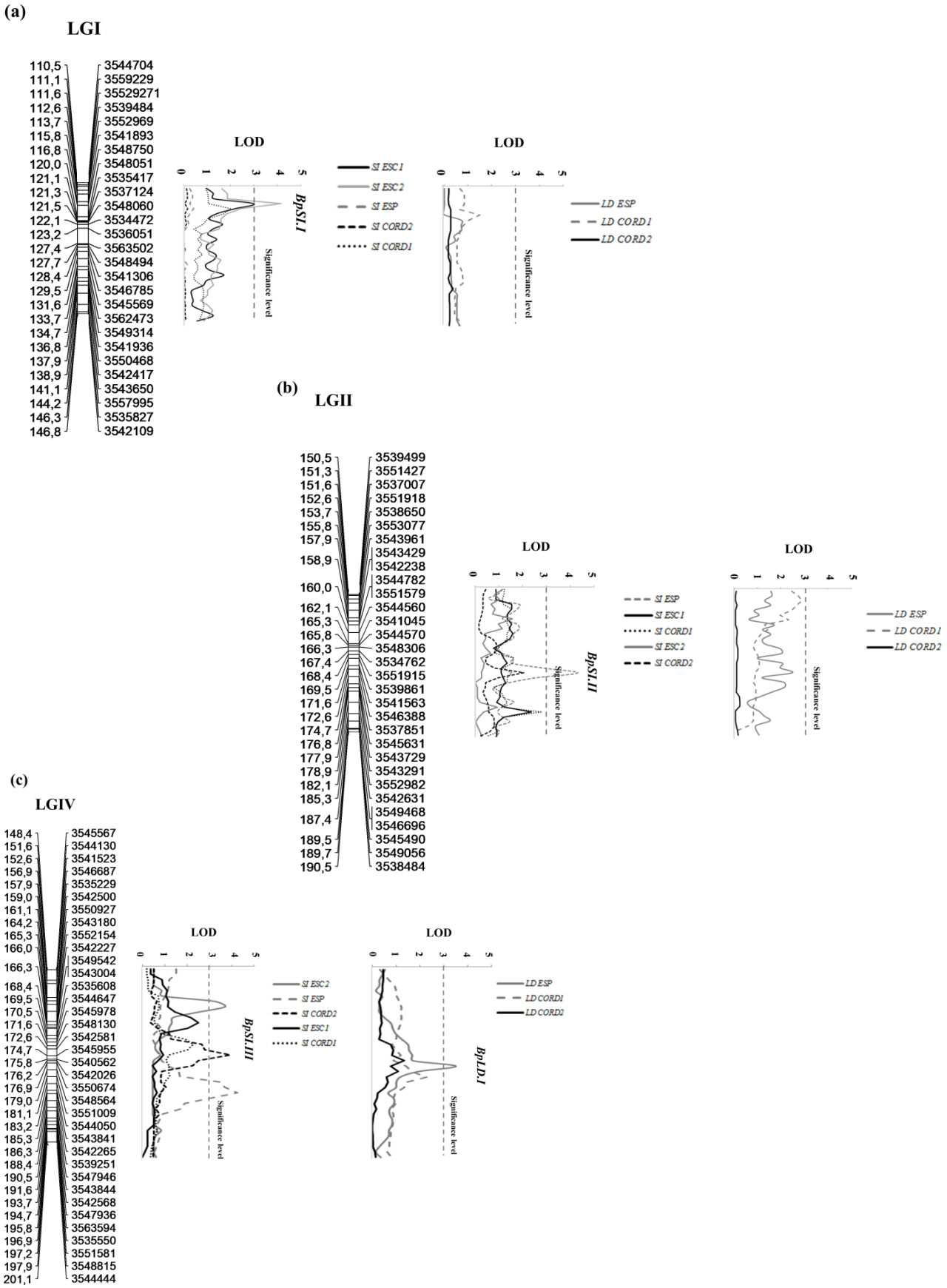

Figure 4. Likelihood plots of consistent quantitative trait loci (QTL) for plant resistance to *Bruchus pisorum* on linkage groups (LG) I (a), II (b), IV (c), using MapQTL in the *Pisum sativum* ssp*. syriacum* (P665) and *P. sativum* ssp. *sativum* (cv. Messire) RIL population. Significant LOD thresholds were detected based on 1000 permutations. Absolute positions (in cM) of the molecular markers along LGs are shown on the vertical axes. SI: % of seed infestation; LD: larval development index.

QTL *BpSI.III*, associated to reduced SI, showed ESC2, ESP and CORD2 with LOD score 3.72, 4.26 and 3.94 explaining 22%, 18% and 18.4 % of the phenotypic variation, respectively (Figure 4, Table 5). *BpSI.III* was localized between 164.22 and 183.2 cM from the beginning of LGIV, between the derived DArT markers 3550927 and 3552154 (for SI ESC2), 3551009 and 3543841 (for SI ESP) and markers 3548130 and 3545955 (for SI CORD2) (Table 5). The distance to the left and to the right flanking markers was of approximatively of 2 cM for each side. Similarly to the previous QTL, peaks in this same region were also detected for SI ESC1 and SI CORD1 (located at 162.1 and 175.8 cM, respectively), explaining 12.7 and 11.5% of the variation, but with no significant LOD scores (2.28 and 2.01, respectively).

A single QTL was identified at LGIV (LOD = 3.51) explaining 16.1% of phenotypic variation for LD at ESP. The peak of the QTL *BpLD.I* was found at 175.8 cM from the beginning of the LGIV, between flanking markers 355955 (1.05 cM) and 3542026 (1.76 cM) (Figure 4, Table 5). In the same region a peak was found in LD CORD1 (located at 176.8 cM, respectively), explaining 7.5% of the variation, but with no significant LOD score (2.28). The third environment evaluated for this trait, did not present any peak.

The resistance-enhancing allele for SI came from the resistant parent P665, as shown by the negative value of the additive genetic effect (ranging from -4.42 to -0.22) (Table 5). By contrary, reduced LD derived from susceptible parent Messire (additive effect = 0.12) (Table 5). Epistatic interactions among QTLs were not significant according to MIM for any of the analysed traits.

4. DISCUSSION

The seed yield and quality losses caused by pea weevil worldwide are reinforcing the need to develop resistant cultivars what is hampered by the scarcity of sources of resistance of the lack of knowledge on its inheritance. Some sources of incomplete resistance are available, but mainly in wild relatives or on non-elite germplasm, forcing for a crossing and thorough selection what is further complicated by environmental influences on insect life cycle and infestations. Availability of molecular markers is most needed to facilitate this selection. Moreover, the possibility to combine resistant genes to different disease in the same cultivar is an added value of the recognition of QTLs associated to pea resistance (Tar'an et al., 2003).

The only previous genetic analysis on resistance to *Bp* was performed in F_2 population from an interspecific cross between *Pisum sativum* x *Pisum fulvum* under controlled conditions. The map was composed of 155 microsatelite markers, separated between them by

an average of 13.3 cM, in 8 LG covering 2,685.8 cM of field pea (Aryamanesh et al., 2013). Those studies identified several regions associated to *Bp* resistance explaining witj 8 QTLs for cotyledon resistance, the 80% of phenotypic variation, and with 5 QTLs and 1 QTL the 70% and 9% of phenotypic variation for seed coat and pod wall resistance, respectively. In this study the map was performed in an advanced RIL F_9 from a *Pisum sativum* species cross with 10,399 markers in 7 linkage groups, and phenotypic data was taken under field conditions. Three QTL (*BpSI.I, II* and *III*) explaining individually between 12.6 to 20.6% of weevil SI variation were identified, and one QTL (*BpLDI*) explaining 16.1% of larval development variation. According to our knowledge, this is the first high-density integrated DArTseq SNP-based genetic map analysis for *Bp* resistance, even more using RIL involving two subspecies, *sativum* and *syriacum* under field conditions.

Data from field phenotype elucidated the high environmental effects on SI and LD, as expected (Aznar-Fernández et al., 2017). Still, resistance of P665 showed lower SI and LD scores in all environments and Pearson correlations showed same tendency for both traits along the environments evaluated (Table 3). The QTLs associated to reduced SI and to high LD were heavily influenced by environment. However, QTL *BpSI.III* (on LG IV) was found in all environments (Figure 4c). RIL population for *BpSI.III* showed skewness towards low SI. Interestingly has been reported that for an interspecific population (*P. sativum* x *P. fulvum*) the low heritability of pod resistance, meanwhile the resistance in the seed lasted (Byrne et al., 2008).

QTL *BpSI.I* (LGI) was clearly detected in three environments and suggested although (LOD < 3) in the remaining two (ESC1 and CORD1) (Table 5, Figure 4a). QTL *BpSI.I* (LGI) was found in only two environments, coinciding with the two trials performed at Escacena. QTL *BpSI.II* (LGII) was identified in one location only (LOD= 4.26 in ESP) although suggested (LOD < 3) in two other environments (ESC1 and CORD1). This shows the high influence of the environment that can affect both the insect and the plant life cycles (Franklin, 2009, Aznar-Fernández et al., 2017). *B. pisorum* dispersion is difficult to monitor and could produce oscillations in the population present in a field. In addition CORD and ESC had a history of *Bp* infestation, which means that the appearance in the field could be staggered as they leave their obligate diapause depending on the environment affecting this way SI values (Table 2). On the other hand ESC environments showed the highest levels of accumulated radiation and temperature average, being able to influence the identification of the QTL (*BpSI.I*).

The environmental discrepancies are unlikely to be due to imprecisions in the assessments, but environmental effects on *Bp* and pea life cycles and their interactions. Seed infestation reduction could be influenced by several factors encompassing multiple plant resistance mechanisms that can act separately or simultaneously. P665 has pigmented flowers and pods, what is associated to condensed tannins (Barbehenn and Constabel, 2011) that might deter *Bp* oviposition (Aznar-Fernández and Rubiales, 2018 a). In addition, possible presence of a thick wax layer or volatiles presence should not be discarded (Chang et al., 2006, Ceballos et al., 2015). This might be affecting SI for which we identified three QTL (Table 5). The previous study conducted by Aryamanesh et al., (2013) broke down the resistance mechanisms that influenced SI, revealing one minor QTL, (POD7) explaining the 8% of phenotypic variation, associated with pod resistance in LG (VII); two major QTL for pod wall/ seed coat resistance explaining the 39% of phenotypic variation, SCR2 and $SCR_{a,b}$ in two different LG (II and V) with additive dominant as well as epistatic effects.

Accession P665 also displayed antibiosis by hampering larval development (Aznar-Fernández et al., 2017). This trait could be affected by a range of compounds that could be toxic for *Bp* larvae. The huge variability along the environments could be possibly linked to the biochemical differences suffered by plants in different environments as commented before. We detected one QTL affected reduced LD in two out of the three environments studied (Figure 4c). Suggesting the possibility of that there is more than one gene in the same QTL explaining both characters on LGIV (SI and LD) (Table 5).

In our study SI resistance, which probably is done by pod antoxenosis, came from the resistant allele (P665) in agreement with Aryamanesh et al., (2013). The only QTL for LD, linked to antibiosis, came from the susceptible parent Messire, on previous studies has been reported cotyledon influence on *Bp* development in both, resistant and susceptible allele (Aryamanesh et al., 2013).

LGs I, II and IV from our saturated *Pisum* map correspond to the LGs 3, 7 and 2, respectively, of *P. sativum* genetic map (Loridon et al., 2005, Bordat et al., 2011, Carrillo et al., 2014). A previous study based on models of segregation, suggest that the complete resistance to *Bp* is controlled by three recessive alleles pwr_1, pwr_2 and pwr_3, with additive effects and dominant epistasis for susceptibility (Byrne et al., 2008). Our QTL for SI (*BpSI.III*) and the only QTL found for LD (*BpLD.I)* were in LG IV, corresponding to LG2 form *P. sativum* where two gens have previously been described for resistance to pea seed-borne yellow mosaic virus and pea

aphid, sbm-2 and mo (Provvidenti and Alconero, 1988). The QTL *BpSI.I* was located in LG3 from *P. sativum*, which encompass two subfamilies of R proteins TIR-NBS-LRR and CC-NBS-LRR, suggesting their implication against *Bp,* as well as for other wounding pests such as pea aphid (*Acyrthosiphon pisum*) in *P. fulvum* (Barilli et al., 2018a). Finally the last QTL found for SI (*BpSI.II)* was correlated with LG7 of *P. sativum*, were the gene ppi2 involved in the resistance to *P. syringae* pv. *pisi* race 2 in pea is closely located (Hunter et al., 2001). Unfortunately we cannot compare our LGs with those of the first study conducted by Aryamanesh et al., (2013) in QTL for Bp resistance, since their did not correspond its map to one of *Pisum sativum*.

In order to state this results a further study should be done to reveal the genes associated to the QTLs found. In addition, to be able to enclose where the resistance mechanism is performed, a greenhouse bioassays such as those developed by Aryamanesh et al., (2013) would be required.

This study reveal one robust QTL associated to reduce SI, as has been identified in the 5 environment, assessed with significant LOD score in most of them (Table 5, Figure 4c). In addition two QTLs associated to low levels of SI have been identified. The identification of genetic markers associated to low pea infestation are of huge interest as repelling the egg laying preserve the pea seed intact. In addition the QTL associated to LD is of great interest in order to prevent this allele, as it is linked to susceptibility. Obtaining QTLs under field conditions is of a wide value at the moment of trusting in the results.

5. ACNOWLEDGMENTS

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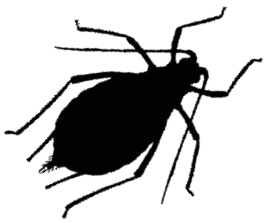
CHAPTER 4

Identification and characterization of antixenosis and antibiosis to pea aphid (*Acyrthosiphum pisum*) in *Pisum* spp. germplasm



Aznar-Fernández T, Rubiales D, 2017. **Annals of Applied Biology, early view,** DOI 10.1111/aab.12417

Category: Agriculture Sci, Multidisciplinary; JCR: **D1**

TITLE:

Identification and characterization of antixenosis and antibiosis to pea aphid (*Acyrthosiphum pisum*) in *Pisum* spp. germplasm

ABSTRACT:

Acyrthosiphum pisum is a polyphagous aphid of major importance on the pea crop to which few resistant cultivars are available. In this study we screened a germplasm collection of *Pisum* spp. under field conditions over two seasons yielding the identification of a number of accessions with intermediate levels of resistance. Selected accessions were further studied under semi-controlled and controlled conditions in no choice and choice assays to validate the responses, and to further characterize the mechanisms of resistance operative. Results elucidated the valuable resistance of accession P40 (*P. sativum* ssp. *sativum*) and P665 (*P. sativum* ssp. *syriacum*), with the combination of both antixenosis, by reducing aphid preference, and antibiosis, by diminishing aphid proliferation.

KEYWORDS: Pea aphid; Plant resistance; Antixenosis; Antibiosis

1. INTRODUCTION

Pea (*Pisum sativum* L.) is a temperate grain legume widely cultivated for animal feed and human food (Rubiales et al., 2009a, Smýkal et al., 2012), being the second most cultivated in the world and the first in Europe (FAOSTAT, 2014). However, pea production can be damaged by a number of diseases and pests (Rubiales et al., 2015a). Pea aphid (*Acyrthosiphon pisum* Harris; Homoptera: Aphididae), hereinafter referred to as PA, is a phloem sucking pest causing major losses in pea crop. PA causes curly and wilting leaves, nutrient deficiencies, plant stunting, reduce the number of seeds and/or pods (Maiteki and Lamb, 1985) and can also act as vector of a number of viruses. Being a polyphagous pest, it can also feed on other legume species (Caillaud et al., 2002, Edwards and Singh, 2006b) such as alfalfa (Gao et al., 2008a), lentil (Andarge and Westhuizen, 2004), lupin and broad bean (Schwartzberg et al., 2011a). This, together with its complex life cycle (Moran, 1992, Blackman and Eastop, 2014) with both sexual and asexual reproduction, and its plasticity for environmental adaptation (Tares et al., 2013, Srinivasan et al., 2014) contributes to its dispersal and survival.

Chemical control is possible but requires repeated treatments, even at the risk of developing resistance to the pesticides (Vanlerberghe-Massutti and Guillemaud, 2007, Bass et al., 2014). Biological control has been attempted with a range of natural enemies (Lagos et al., 2001, Snyder and Ives, 2003), although with insufficient results due to the complexity in maintaining the needed balanced system. Intercropping studies have shown a reduction of PA infestation and the improvement of biocontrol (Seidenglanz et al., 2011, Ndzana et al., 2014) but also without the required efficiency. Therefore, studies for develop and use of resistant cultivars to PA is most desirable (Gu et al., 2008). Although some levels of incomplete resistance have been reported (Newman and Pimentel, 1974, Bieri et al., 1983, Soroka and Mackay, 1991, Ali et al., 2005) and some progress has been achieved in understanding defence mechanisms (Carrillo et al., 2013, Mai et al., 2014), this has not yet resulted in the release of resistant cultivars. This reinforces the need to identify additional sources of resistance to be used in pea breeding programs.

The objective of this study was to identify new sources of resistance and to characterize the mechanisms of resistance operative, with the goal to combine them by breeding in elite germplasm to achieve a more durable resistance against PA.

2. MATERIAL & METHODS

2.1. Germplasm collection screening under field conditions

A germplasm collection consisting of 69 *Pisum* spp. accessions (Table 1), maintained at the Institute for Sustainable Agriculture, Spain, and earlier provided by USDA, USA (PI-numbers), John Innes Centre, UK (JI-numbers), Plant Genetic Resources, Holland (CGN-numbers) or ICARDA, Syria (IFPI-numbers), was screened for PA resistance under field conditions at Córdoba, Spain, during seasons 2013-2014 and 2014-2015. Climatic data is provided in Table 2 for both growing seasons, from December to June (obtained from: http://www.juntadeandalucia.es/agriculturaypesca/ifapa/ria/servlet).

Table 2. Climatic data of two growing seasons evaluated. The parameters (Tmax, Tmin: absolute maximum and minimum temperatures, respectively; Av. T: average temperature; Av. humidity: average humidity; Rainfall: accumulated rainfall; Av. Rad: average radiation) are given for the period between sowing and harvesting from 2013 to 2015. Flowering period started on March the first season and on February the second season.

Season	Period	Tmax (ºC)	Tmin (ºC)	Av.T (ºC)	Av. Humidity (%)	Rainfall (mm)	Av. Rad (W/m2)
2013-2014	Vegetative	20.2	-3	7.8	84.33	296	8.4
	Flowering	31.1	1.8	14.9	72.5	83.2	19
	Reproductive	38.4	7.9	22.0	55.5	24.4	27.8
2014-2015	Vegetative	20.2	-3.3	7.3	86.0	60.4	9.18
	Flowering	31.2	0.3	13.2	73.4	111.2	19.6
	Reproductive	42.8	8.6	23.8	44.3	8.9	27.7

Latitude: º37º 51' 25'' N; Longitude: º04º 48' 10'' W; Altitude: º117.0

Trials followed a randomized block design with 3 replicates; each pea accession was represented by 5 plants per row (0.5m long, spaced 70cm). Susceptible cv. Messire was included as check. Natural infestations are known to be common in the site; however, in order to ensure a sufficient pest pressure, plots were artificially infested 100 days after sowing, by gently delivering 2 PA nymphs of late instar in the apical part of each plant with the help of a paint brush. Because of the rain (Table 2), first plot assay (2013-2014) was infested a second time after a week following the same procedure as above. Aphid nymphs were collected 24h before by an insect sucker, and placed in darkness at 4ºC. Aphids used derived from a single-

aphid, isolated from a population collected in pea fields at Córdoba in 2013. The clone was maintained on broad bean (*Vicia faba* cv. Brocal) plants, under growth chamber conditions (20 ± 2ºC; 65% RH and 12:12h D:N). The same clone was used in all experiments.

Plots were protected with anti-birds mesh and were drip irrigated. No pesticides were applied on the experimental plots or surrounding fields during the experiments, and were mechanically and manually weeded. Observations started 7-10 days after infestation (dai), and then were repeated weekly by visually estimating the aphid infestation coverage (%AI) of each row. Plant damage (PD) was also estimated the first season (2013-2014) by using a 0-5 visual scale modified from (Carrillo et al., 2013) as follows: 0=no visual symptoms; 1=slight leaflet curl, initial chlorosis; 2=chlorosis and curling in leaflets, molasses cover slightly the plants surface; 3=wilting of apical part, heavily curly leaves and covered by molasses and mildew; 4=> 50% wilting; 5=death. Both %AI and PD data were submitted to an area under plant aphid infestation progress curve (AUAIPC) and an area under plant damage progress curve (AUPDPC), respectively, with the objective to integrate in the evaluation the first data collection till the last one (Jeger and Viljanen-Rollinson, 2001), by using the following formula:

$$AUAIPC\ or\ AUPDPC = \sum_{i=1}^{n-1} \frac{Y_i + Y_{i+1}}{2} \times (t_{i+1} - t_i)$$

where Y is the AI or PD level at assessment date, i is the number of days after the first observation on assessment date and n is the number of successive observations. Because the longevity of pea genotypes differs, data was rescaled by dividing the area of each genotype by the total days of evaluation for each, RAUAIPC and RAUPDPC. In order to facilitate data interpretation, Table 1 showed RAUAIPC and RAUPDPC expressed as relative to the susceptible check cv. Messire (=100%; rRAUAIPC and rRAUPDPC To follow a normal distribution, data for rRAUAIPC and rRAUPDPC were transformed with $\arcsin\sqrt{rRAUAIPC\ or\ rRAUPDPC/100}$ formula.

In addition, plant phenology was studied weekly by assessing the flowering in each replicate. There after the number of days until the first flowers appearance (DFF) days until 50% of flowering (DF) and days of first pod appearance (DFP) in each replicate was estimated with respect to the number of days from sown.

After field screenings a reduced number of accessions were selected for further studies in choice and non-choice screenings under semi-controlled and controlled conditions. These were the *P. sativum* ssp. *sativum* accessions P40 and P29; and the *P. sativum* ssp. *syriacum*, P665. The cultivar Messire was included as susceptible check.

2.2. Validation and characterization of responses on field selected accessions

A first assay was performed on the 3 selected accessions together with cv. Messire as susceptible check under semi-controlled conditions. Seedlings were sown in mid-May in square pots (15x15x15cm filled with 1:1 sand: peat mixture), separated by a minimum of 50 cm under an anti-thrip mesh protected shelter. The experimental design consisted on 5 randomized replicates, each one consisting in a pot with two seedlings of the same accession. Three weeks after planting, seedlings were supported with a thin metal cane and 24h later were infested by gently placing with a paint brush 5 PA nymphs on the apical part of each plant. Nymphs' collection and origin were the same as indicated before (see 2.1). First winged aphids were observed by 10 dai and were not removed in order to simulate PA normal spread under natural conditions (Pettersson et al., 2007). Assessments started 1 dai and were repeated at three days intervals. Aphid development was assessed on the top 4 leaves by counting the number of nymphs from 1 to 8 dai, when apical parts were overcrowded. Moreover %AI was estimated from 1 to 16 dai also in the apical part. Plant damage was assessed by visually estimating % plant wilting (1 dai till 19 dai) and % leaf chlorosis (1 dai till 16 dai). In order to assume a normal distribution data was transformed: nymphs' number and % chlorosis by square root and % wilting by $\arcsin\sqrt{\%wilting/100}$.

The same accessions were also submitted to a multiple-choice assay in larger pots (24cm diameter and 26cm deep) under the same mesh protected shelters. One seedling of each of the accessions was planted along the perimeter of each pot at random distribution, with 10 replications, each one formed by a pot. Three weeks old seedlings were supported individually with thin metal canes. The following day, 60 wingless PA adults were gently placed in the centre of each pot with the help of a paint-brush at dawn to avoid light preferences (Ali et al., 2005). Adults were used for this experiment in order to ensure a faster mobility. Numbers of adults per plant were recorded at 1, 3, 7 and 24 hours after infestation (hai). Thereafter, for each evaluation time the percentage of PA over each genotype was calculated. Data was transformed by square root in order to follow a normal distribution.

In addition, five aphid-free pots were grown under the same conditions in a separate cage, as controls of plant performance.

2.3. Choice and no-choice assays on two selected accessions

2.3.1. Dual-choice assays

A first dual-choice assay with accessions P40 and P665 *vs* the susceptible check cv. Messire was performed on seedlings in pots under the mesh protected shelters described above (section 2.2). A seedling each of P40 or P665 was faced with a seedling of Messire, by sowing them in opposite extremes of pots (15x15x15cm). A total of 10 replications were carried out for each combination. The procedure for infestation and evaluation was the same as described above for multiple-choice assay, but at lower insect pressure by placing 20 PA in the centre of each pot.

A second dual-choice assay was performed on detached leaves of the same accessions under controlled conditions (20 ± 2ºC; 65% RH and 12:12h D:N). Seedlings were previously grown in pots under growth chamber conditions and when they were 3 weeks old, the youngest fully expanded leaves were cut with scissor and gently placed adaxial side up inside Petri dishes filled with 5% water-agar. Two leaves were placed per dish, forming all the possible pair combinations (665 *vs* Messire; P40 *vs* Messire and P40 *vs* P665) with 20 replications for each one. Next, 10 PA nymphs were gently placed in the centre on each petri dish with a paintbrush. Dishes were covered with filter paper during the entire assay to prevent the possible influence of light on the aphids' behaviour. Numbers of PA feeding over each leaf were counted at 1, 2, 4 and 24 hai. New born nymphs and death aphids were not recorded.

2.3.2. No choice assays

Seedlings of accessions P40 and P665 together with the susceptible check Messire were studied in a no-choice assay under growth chamber conditions (20 ± 2ºC; 65% RH and 12:12h D:N) in small pots (7x7x8cm), one accession per pot. The assay consisted of a randomized block design with 6 replications, each one formed by a plastic tray where pots with all the accessions were present. With the objective to study the capacity of PA to cope plant initial barriers (Tjallingii, 2006, Will et al., 2007) and also the defensive response reactions of pea plants, a same aphid pressure was applied. For this, three weeks old seedlings

were supported with a thin metal cane, in order to prevent contact among plants and to reduce potential aphid shift between accessions, and 48h later aphid infestation was performed by gently placing 5 PA nymphs from the same clone as before, on the apical part of each seedling with the help of a paint brush. The possibility of nymphs' movement from one pot to another was reduced by covering the bottom of the trays with soap-water. This was further prevented by daily checking the plants and removing any possible winged adults, with the objective to monitor how same PA pressure developed and affected each accession. A control tray with free-aphid pots of the three accessions was placed under the same chamber conditions. Numbers of nymphs and adults were counted and %AI estimated at 3 days interval till 18 dai. In this trial also % of leaf chlorosis was estimated as a possible mechanism of resistance to aphids (Pegadaraju et al., 2005, Carrillo et al., 2013). Data of number of nymphs, adults and aphid infestation percentage were transformed by arsin, in order to follow a normal distribution.

2.4. Statistical Analysis

Statistical analyses were developed using Statistix 10 ® (Analytical Software, Tallahassee, USA). To analyse field data a combined analysis of variance was conducted to determine genotype and genotype x season interaction for rRAUAIPC. In no choice assays the combined analysis of variance was conducted to determine the genotype, time of evaluation and treatment (checking the tested accessions and the controls) for each assessment. Pearson correlation was calculated to study the possible relationship between plant phenology (DFF, DF and DFP) over rRAUAIPC and rRAUPDPC.

3. RESULTS

3.1. Germplasm screening under field conditions

Responses of pea germplasm evaluated against PA attack under field conditions are shown in Table 1. Data are referred to the control Messire (set as 100%) with the aim to facilitate data interpretation and comparison among seasons. ANOVA for rRAUAIPC (Table 3), revealed significant effect of genotype (G), season (S) and GxS interaction ($P<0.05$), being the highest mean of square for S, followed by far for G and the lowest for the interaction GxS. Higher infestation levels were achieved in 2013-2014 with an average of 28.3% on Messire

check, being of 2.6 in 2014-2015. A number of accessions displayed significantly lower levels of infestation than the check Messire in both seasons, maintaining the same relative ranking. Nevertheless in the first season, the higher infestation levels largely reduced differences with Messire for rRAUAIPC. In the first season highlighted the 10.0% for P659 followed by P658 and P29 (44.0 and 56.6%, respectively), whereas 44 accessions were closed to Messire in the range of 87.4-71.2%. The second season 2014-2015, 39 pea accessions displayed significantly lower levels for rRAUAIPC than Messire (in the range of 4 to 72.7%).

Table 3. Analysis of variance for *Acyrthosiphon pisum* coverage (rRAUAIPC) and plant damage (rRAUPDPC) of the 69 pea genotypes in the two seasons evaluated. (DF: degrees of freedom; MS: mean square; G x S: term of genotype x season interaction).

	Source	**DF**	**SS**	**MS**	**F**	**P**
rRAUAIPC	Genotype (G)	68	9.61	0.14	2.47	0,001
	Season (S)	1	7.32	7.32	127.99	0,001
	G x S	68	6.27	0.092	1.61	0,005
	Error	229	13.09	0.06		
	Total	366				
rRAUPDPC	Genotype	62*	8.33	0.14	2.68	0.001
	Error	108	5.41	0.05		
	Total	170	13.75			

* There were 6 missing genotypes for plant damage assessment.

PD was recorded only during 2013-2014 season. There were significant differences in rRAUPDPC (P=0.001), see Table 3; with fifteen accessions showing significant values lower than Messire, being particularly low for P29 (27.7%) and P40 (26.7%). Pearson correlations showed a significant low association between assessments, rRAUPDPC and rRAUAIPC (r=0.345; P=0.006; R^2=0.11).

Concerning correlations between rRAUAIPC (aphid infestation) and plant phenology, DFF revealed a significantly little negative association (r=-0.374; P=0.001), meanwhile DF and DFP showed no significant association (P>0.05). Regarding correlations between rRAUPDPC (plant damage) and DFF, DF or DFP, calculated with field data from the first season (2013-2014), showed no significant association (P>0.05).

3.2. Validation and characterization of responses on field selected accessions

Assessments carried out in no choice assays under semi-controlled conditions are shown in Figure 1, corroborating field data. ANOVA showed significant differences ($P<0.05$) for the four traits assessed in the evaluated accessions (Table 4). Number of nymphs, % AI and % wilting were heavily influenced by days of evaluation, followed by the genotype.

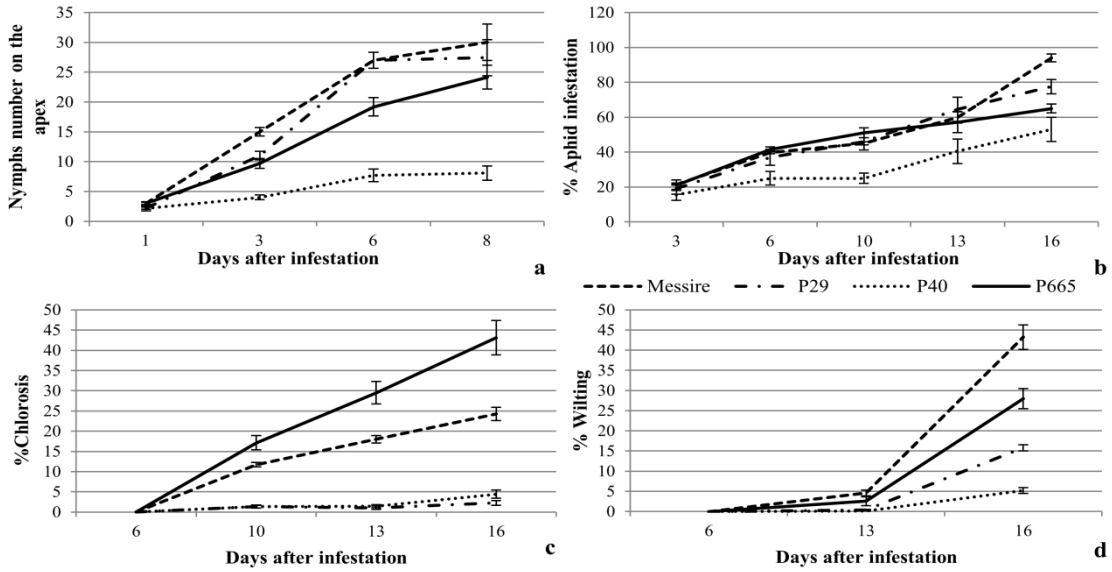


Figure 1 Validation and characterization, under semi-controlled conditions, of responses to pea aphid in the selected pea accessions. a) Number of nymphs counted on the apical part of plantings; b) Percentage of aphid coverture on the apical part of plantings; c) % of chlorosis on seedlings; d) % of wilting on seedlings. Bars = standard error of the mean.

Susceptible check cv. Messire showed the highest levels of infestation with the highest number of nymphs per plant (30 after 8 days of infestation) and the highest %AI (94% 16 dai). As a result of this infestation, Messire showed moderate levels of chlorosis (24% by 16 dai) and the highest values of wilting (43% by 19 dai). Accession P40 showed the lowest infestation as shown by both a lower number of nymphs (8 on the last assessment) and lower %AI (53% by 16 dai) and also showed the lowest level of plant damage at all observation times. Accession P29 showed similar values of AI than Messire at all time points, but suffered less PD both in terms of chlorosis and wilting. Accession P665 showed moderate to high number of nymphs on the apex, however at 16 dai a reduction of %AI (65%) could be observed. On the other hand P665 showed the highest % of chlorosis although only moderate % of wilting. Controls sown under the same semi-controlled conditions showed no PD.

Results of multiple choice assays carried out under semi-controlled conditions are shown in Figure 2. ANOVA showed significant genotype and hours after infestation influence (Table 4). Data confirmed that pea cv. Messire was the preferred accession along the entire assay. Nevertheless general tendency was that P40, P29 and P665 were less preferred, with little differences among them, being P665 significantly less preferred most of the time.

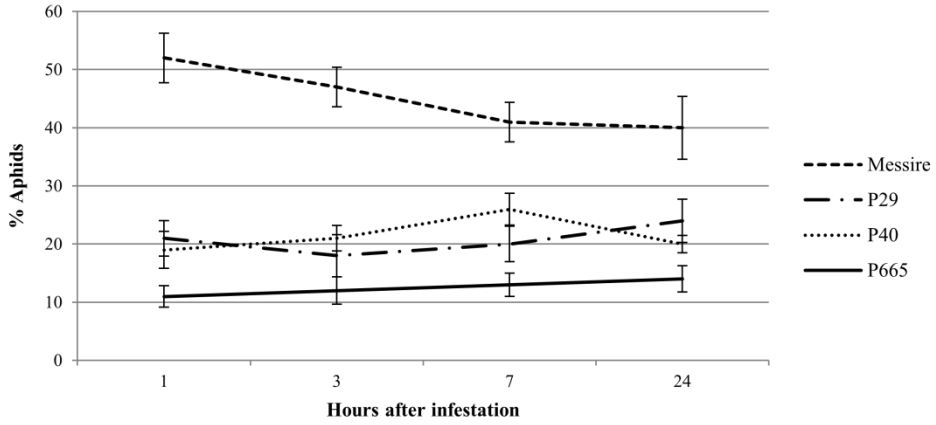


Figure 2 Percentage of pea aphids on selected pea accessions in multiple choice assay along time under semi-controlled conditions. Bars = standard error of the mean.

Table 4. Analysis of variance from assays under semi-controlled and controlled conditions. *Acyrthosiphon pisum* development and plant performance on pea genotypes selected. (DF: degrees of freedom; MS: mean square; G x D: term of genotype x days after infestation (dai) or G x H: hours after infestation (hai)).

		Source	DF	SS	MS	F	P
NO CHOICE ASSAYS	SEMI- CONTROLLED CONDITIONS						
	Nymphs number on the apex	Genotype (G)	3	60	20	15.09	0.001
		Days after infestation (D)	3	187	62	47.37	0.001
		G x D	9	19.26	2.14	1.63	0.113
		Error	140	184.24	1.32		
		Total	155				
	% Aphid infestation	Genotype (G)	3	4923	1640.9	5.82	0.001
		Days after infestation (D)	5	160253	32050.7	113.66	0.001
		G x D	15	9541	636.1	2.26	0.005
		Error	269	75852	282		
		Total	292				
	% Chlorosis	Genotype (G)	3	295.67	98.56	47.95	0.001
		Days after infestation (D)	2	38.94	19.47	9.47	0.0002
		G x D	6	9.77	1.63	0.79	0.579
		Error	90	185	2.06		
		Total	101				
	% Wilting	Genotype (G)	3	1.02	0.34	16.47	0.001
		Days after infestation (D)	2	5.33	2.67	129.1	0.001
		G x D	6	0.96	0.16	7.78	0.001
		Error	99	2.04	0.02		
		Total	110				
		Source	**DF**	**SS**	**MS**	**F**	**P**
	CONTROLLED CONDITIONS						
	Nymphs number	Genotype (G)	2	302.168	151.08	74.76	0.001
		Days after infestation (D)	2	166.224	83.11	41.12	0.001
		G x D	4	51.978	12.99	6.43	0.001
		Error	45	90.944	2.02		
		Total	53	611.314			
	Adults number	Genotype (G)	2	0.29	0.14	61.55	0.001
		Days after infestation (D)	3	0.11	0.04	15.98	0.001
		G x D	6	0.12	0.02	8.42	0.001
		Error	60	0.14	0.002		
		Total	71	0.66			
	% Aphid infestation	Genotype (G)	2	0.51	0.25	17.55	0.001
		Days after infestation (D)	2	0.79	0.40	27.38	0.001
		G x D	4	0.19	0.05	3.34	0.018
		Error	42	0.61	0.01		
		Total	50				

		Source	DF	SS	MS	F	P
CHOICE ASSAYS	Multiple Choice assay	Genotype (G)	3	34.63	115.42	30.8	0.001
		Hours after infestation (H)	4	3.63	0.91	2.42	0.05
		G x H	12	3.31	0.28	0.74	0.714
		Error	180	67.45	0.37		
		Total	199	109.02			
	Dual choice assay (semi-controlled conditions)	Genotype (G)	1	2420.1	2420.1	8.16	0.006
		Hourss after infestation (H)	4	553.9	138.5	0.47	0.760
		H x D	4	1585.8	396.4	1.34	0.266
		Error	65	19270.5	296.5		
		Total	74				
	Dual Choice assay (controlled conditions)	Genotype (G)	3	7120.79	2373.6	2.87	0.037
		Hours after infestation (H)	4	1.37E-23	3.44E-24	0	1
		G x H	12	1588.93	132.411	0.16	1.000
		Error	280	231425	826.516		
			299	240134			

Pearson correlations elucidated a strong association between data from multiple choice assay 24 hai and data from %AI on the apical part of pea plants taken 16 dai (under winged aphid presence) (r=0.98; P=0.001).

3.3. Choice and no-choice assays on two selected accessions

3.3.1. Dual-choice assays

ANOVA for dual choice assays under semi-controlled and controlled conditions (Table 4) showed genotype influences; meanwhile hours after infestation did not, for this reason Figure 3 shows percentage of PA over each accession in the last assessment date (24 hai).

Accessions P665 and P40 were significantly less preferred than the check Messire in both assays. Under semi controlled conditions (Figure 3a) there was a 24% and 40% reduction of %AI for P40 and P665, respectively. This reduction was a bit smaller in detached leaves under controlled conditions (Figure 3b), being of 16 and 22% for P40 and P665, respectively. When P665 was confronted with P40, it was less preferred by 10%.

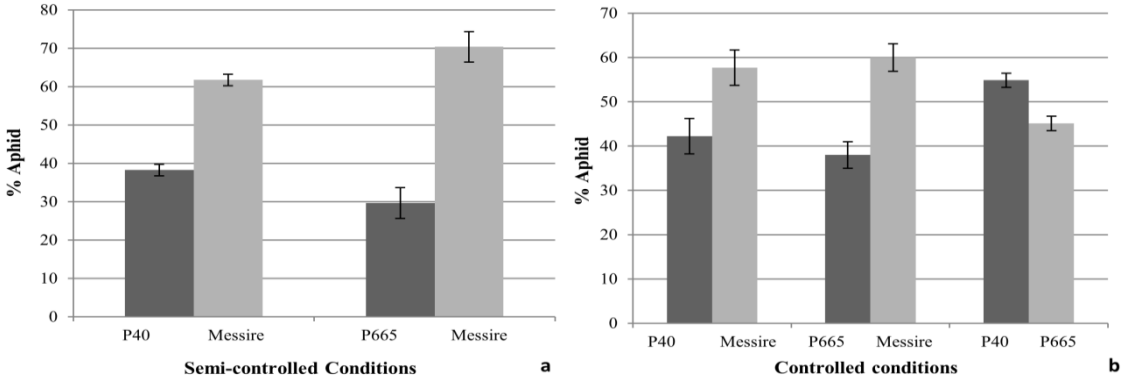


Figure 3 Distribution of pea aphids by 24hai in dual choice assays. a) Seedling under semi-controlled conditions; b) Detached leaves under controlled conditions. Bars indicated mean ± standard error of the mean.

3.3.2. No choice assay

Results showed significant differences ($P<0.05$), for the assessments carried out in no choice assays under the same PA pressure and corroborate data under semi-controlled conditions (Table 4). Messire showed the highest values of nymphs (Figure 4a), number of adults (Figure 4b) and %AI (Figure 4c). P40 showed the lowest %AI at all assessment times which is translated into also showing the lowest number of adults and the lower number of nymphs. P665 did not differ significantly from Messire in %AI, in spite of a significantly lower numbers of adults and of nymphs already from 11 hai. This might be explained by the smaller biomass of P665, with smaller leaves so the actual reduction in numbers of aphids might be diluted when assessing % plant canopy covered by aphids, as was the case here, with a reduced but not significant %AI.

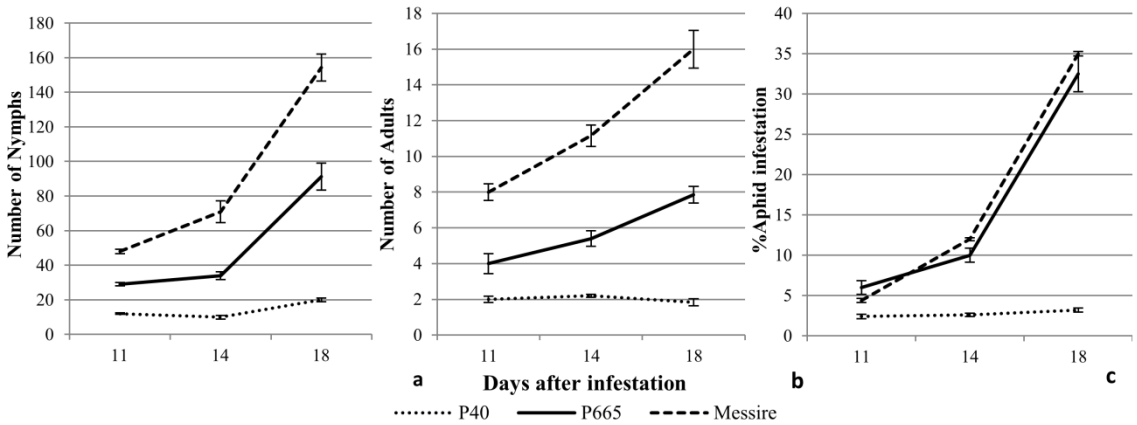


Figure 4 Comparison of pea aphid development on selected accessions in no-choice assay under controlled conditions. a) Number of PA nymphs; b) Number of PA adults; c) % Aphid infestation. Bars = standard error of the mean.

Regarding PD assessed by chlorosis evaluations (Figure 5), factorial analysis for %chlorosis showed the influence of tested and controls plants, genotype and dai (P=0.001). Messire, control and infested, showed intermediate levels of %chlorosis, shared with P665 control, meanwhile infested P665 displayed the heaviest %chlorosis levels. On the other hand, P40, with the lowest PD values, showed no significant chlorosis differences between infested and control seedlings.

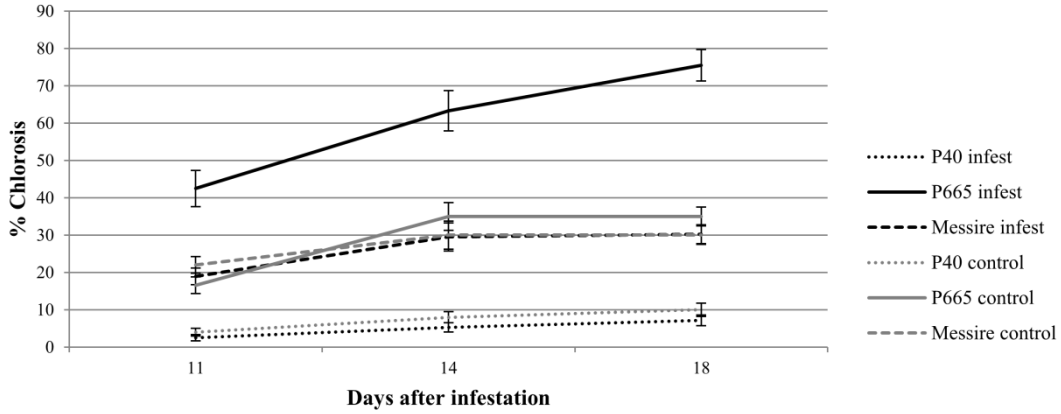


Figure 5 Plant chlorosis in infested (Infested) and non-infested (Control) pea accessions with pea aphids, under chamber conditions. Bars = standard error of the mean.

The set of results obtained under controlled conditions showed the lowest PD and %AI jointly with reduced PA reproduction and lowest PA feed preference for accession P40, meanwhile accession P665 stood out due to low PA infestation and no PA preference. For additional information about phenology of each accession see Table 5.

4. DISCUSION

Resistance to PA is a priority in pea breeding. Although some levels of incomplete resistance to PA has been described (Newman and Pimentel, 1974, Bieri et al., 1983, Soroka and Mackay, 1991, Ali et al., 2005) this has not yet resulted in the release of resistant cultivars. In an attempt to identify additional sources of resistance we screened a *Pisum* spp. germplasm collection including landraces and wild relatives under field conditions. This allowed the identification of varying levels of resistance that were further validated under semi-controlled and controlled conditions. There was season influence on aphid infestation levels, with a lower infestation in 2015 than in 2014. This might be due to the second infestation in the field, promoted for the continued rainy days after onset of infestation in 2014. Additionally 2013-

2014 season suffered lower temperatures and reduced rainy days during flowering time (Table 2) fostering PA development. However, genotypic effects were significant allowing the identification of accessions with reduced infestation. This might be due to a wide range of responses such as escape (no coincidence of the pest with the proper state of the plant to be infested), antixenosis (low preference of the pest to certain accessions) and antibiosis (capacity of the plant to block the biology of the pest and reduce its number by interfering in its reproduction). These mechanisms could act alone or in combination (Mendesil, 2014). In addition, the little association between AI (rRAUAIPC) and PD (rRAUPDPC) in the field suggests the possibility of tolerance to aphid damage in the pea collection evaluated. Growth stage of the plant is known to influence aphid reproduction and damage (Maiteki and Lamb, 1985). Infestation at flowering might be particularly damaging as flowering is a critical period for grain yield determination (Sandaña and Calderini, 2012), being very sensitive to PA attack (Ofuya, 1989). Our field experiments were designed to quantify PA infestation on the whole plant canopy, what could be affected by precocity, biomass as well as by genetic resistance. Specific bioassays allowed confirming resistance of selected accessions and to discerning about possible antixenosis and antibiosis mechanisms. We found little correlation between precocity and infestation in the collection, but cannot exclude this possibility on specific accessions, as the germplasm was highly variable in phenology and morphology. Both pea flowering and aphid development are influenced by light, nutrients and water availability; factors that could change every season (Levy and Dean, 1998) and might cause escape of certain varieties. In addition at higher PA infestation the competition for food is bigger (Randolph and Randolph, 1977) what might be affected by blooming, when amino acid composition is lower (Maltais and Auclair, 1957, Auclair et al., 1957). We can neither exclude existence of genetic variation for resistance to infestation in pods, being independently from infestation on canopy. Further specific experiments should be designed to discern these possibilities.

With the objective to discern the presence of possible antixenosis and antibiosis mechanisms, we further characterized the responses of selected pea accessions in choice and non-choice assays under semi and controlled conditions. The set of results suggested a combination of both antixenosis and antibiosis mechanisms in P40 and P665 accessions. Such combination of mechanisms was previously reported in pea (Soroka and Mackay, 1991, Ali et al., 2005) or in soybean to soybean aphid (Diaz-Montano et al., 2006).

Data from multiple choice and non-choice assay under semi-controlled conditions was submitted to a Pearson correlation, to disclose if winged (present in no choice, %AI) and

apterous (present in multiple choice, %Aphids) PA shared the same host preferences. Results revealed a strong correlation between both assessments suggesting the coincidence between both types of aphids in the selection of a host plant, which reinforces our results.

Choice assays showed that the selected accessions were less preferred by PA than Messire, as already seen by 1 hai, and maintained at all time points (Figure 2). It is known that phloem ingestion by PA starts around 2 hai on fava (Mutti et al., 2008b), by this we suggest that the three pea accessions might display antixenosis as a resistant mechanism. Antixenosis has been reported to be contributed by a number of factors: colour of foliage (Cartier, 1963), odour and plant volatiles (Hegde et al., 2012, Jaquiery et al., 2012), glandular trichomes (Peter, 1995), surface wax bloom (White and Eigenbrode, 2000, Chang et al., 2006) or leaf surface disposition and conditions, which determine grip and therefore PA locomotion through the plant (Friedemann et al., 2015), among others. Due to the high number of factors that may be involved, we cannot conclude on the mechanistic reason for antixenosis in our accessions. Moreover, P29 and P40 shows pigmented flowers and P665 shows creeping growth habit and has violet flowers and red pigmentation on pods (Table 5). This pigmentation might be done by condensed tannins or flavonoids, which have been associated with resistance to aphids in cowpea (Grayer et al., 1992) and black bean (Lattanzio et al., 2000), and suggested as antifeedant metabolite to PA (Golowska and Lukasik, 2012). On the other hand P40 showed low PD, which might contributed to growth tolerance to PA in field, since the tissues of a mature plant, if it is healthy, are less tender and therefore a greater part of the plant becomes less palatable for aphids.

Regarding non-choice assays, some levels of antibiosis are also suggested for accessions P665 and P40. P665 displayed intermedium number of nymphs and adults under no choice assay. A previous study (Carrillo et al., 2013) pointed the moderate resistance of this accession associated with the activation of different metabolic pathways resulting in a reduction of the amino acids available and the photosynthetic activity, which corroborate our data with the higher chlorosis values reached by P665. Such nutrients reduction might be counter-productive for PA development; as reported with senescence in aphid-infested tomato (Pegadaraju et al., 2005). As a resistant accession, P665 might be able to counteract self-inflicted damage stimulated by aphid presence likewise auto toxicity, chlorosis or photosynthesis modification. Contrary to P665, no PD was observed in P40 but showed the lowest number of nymphs and adults and subsequently the lower AI rates. This suggest antibiosis acting at phloem level (Klingler et al., 1998) as result of limited availability of

nutrients required by PA (Pedigo, 1989) or resistance genes expression associated to aphids damage (Smith and Boyko, 2007), which hampers PA development. Antibiosis has been already reported in pea (Soroka and Mackay, 1991), red clover (Zeng et al., 1994) and barrel medic (Gao et al., 2008a). In order to be able to decode at molecular levels how the antibiosis mechanism in P40 works, proteomic and transcriptomic studies are being performed and will be presented elsewhere.

Accession P29 showed low PD in spite of high AI levels (similar to Messire), although more studies are needed to discern the mechanism involved, data suggest a tolerance response by counteracting the PA stress without overcoming it. The involvement of phytohormones in plant tolerance to several pests has been revealed, such as jasmonic acid (JA) in Arabidopsis (Abe et al., 2008, Ellis et al., 2002) or JA, auxin and ethylene (ET) in wheat (Smith et al., 2010). Tolerance mechanism is considered as a powerful defence mechanism, since it does not affect yield, delays insecticidal treatments (Smith and Chuang, 2014) and makes the biocontrol a compatible treatment to reduce the pests. However, the non-eradication of the pest compromises the safety of other crops.

The results of our study suggest two potential pea accessions, P40 and P665, with a combination of antixenosis and antibiosis resistance. Being P40 a *Ps. sativum*, it possesses suitable agronomic qualities for farmers, such as high amount of pods with 6-7 seeds and prolonged growth, and will undoubtedly increase cross-breeding success. On the other hand, P665 (*P. sativum* ssp. *syriacum*) is also readily used in pea breeding as crosses are feasible and have readily been used in pea breeding (Rubiales et al., 2009b). An added value to accession P665 is that it's know to carry other resistances, such as to pea weevil (*Bruchus pisorum*) (Aznar-Fernández et al., 2017), ascochyta blight (*Didymella pinodes*) (Fondevilla et al., 2005), broomrape (*Orobanche crenata*) (Rubiales et al., 2005b) and also presents drought adaptation (Iglesias-García et al., 2015) making it a desirable candidate in plant breeding programs. On the other hand possible tolerance to PA has been elucidated in P29, being also a *P. sativum* ssp. *sativum* which makes its resistance readily available for pea breeding.

In addition the identification of new resistant sources against the PA model, allowed the development of new assays to amplify insect-interaction knowledge. Moreover both resistances described are incomplete, which suggests a complex trait inheritance, complicating for PA to overcome P40 and P665 resistances (although genetic analyses are needed before this can be stated).

5. ACNOWLEDGMENTS

Assistance of Javier García and Ana Álvarez for technical support and Dr. Angel Villegas for the helpful comments on the manuscript are acknowledged. Research was supported by Spanish Ministry of Economy and Competitiveness grant BES-2012-061849 and project AGL2014-52871-R, co-financed by FEDER and by EU project FP7-LEGATO.

Table 1. Response to pea aphid attack of 69 *Pisum* accessions studied under field conditions during two seasons (2014-2015). rRAUAIPC (rescaled area under aphid infestation progress curve), rRAUPDPC (rescaled area under plant damage progress curve). All data are referred to the control Messire (Messire =100%). Accessions are ordered from lowest to highest rRAUAIPC value from 2014-2015.

						2013-2014						2014-2015		
	Accession	Synonym	Origin	Species	Subspecies	rRAUAIPC ± SEM			rRAUPDPC ± SEM			rRAUAIPC ± SEM		
P	671	IFPI 3338	Turkey	*sativum*	*elatius*	73.8	±	9.08		m		4.4	±	1.64
P	648	IFPI 2372	Ethiopia	*sativum*	*arvense*	83.9	±	2.79	78.6	±	8.09	10.3	±	5.09
P	40	PI 505112	Greece	*sativum*	*sativum*	68.0	±	4.62	26.8	±	8.80	10.5	±	8.34
P	29	PI 505127	Albania	*sativum*	*sativum*	56.6	±	12.66	27.7	±	15.19	15.3	±	5.74
P	651	IFPI 3232	Syria	*fulvum*		68.8	±	8.21	86.9	±	0.62	17.1	±	11.91
P	53	JI 813	USA	*sativum*	*elatius*	75.9	±	4.70	75.4	±	9.11	17.5	±	14.14
P	675	IFPI 3358	Turkey	*sativum*	*elatius*	68.7	±	1.01	95.3	±	6.64	17.9	±	6.81
P	1123	PI 347321	India	*sativum*	*sativum*	81.3	±	5.07	74.4	±	6.76	20.5	±	12.35
P	658	IFPI 3253	Syria	*fulvum*		44.0	±	4.06		m		21.2	±	9.22
P	639	IFPI2363	Ethiopia	*sativum*	*arvense*	78.3	±	10.81	86.3	±	8.29	23.2	±	4.09
P	621	IFPI 436	UK	*abyssinicum*		71.2	±	11.07	69.6	±	3.68	23.9	±	6.76
P	1412	PI 357006	India	*sativum*	*sativum*	81.8	±	4.49	70.2	±	7.76	26.4	±	4.52
P	712	PI 261660	Netherland	*sativum*	*sativum*	79.1	±	3.93	69.6	±	3.73	28.1	±	6.25
P	672	IFPI 3341	Turkey	*sativum*	*elatius*	73.5	±	7.29	85.2	±	15.63	29.2	±	15.76
P	28	PI 505122	Albania	*sativum*	*sativum*	70.3	±	3.55	52.9	±	9.91	30.4	±	14.57
P	669	IFPI 3330	Turkey	*sativum*	*elatius*	74.8	±	5.41	91.7	±	8.39	30.7	±	14.50
P	635	IFPI 2359	Ethiopia	*sativum*	*arvense*	85.5	±	8.83	78.0	±	4.92	31.7	±	6.66
P	19	PI 344006	Greece	*sativum*	*elatius*	67.2	±	13.10	79.3	±	11.04	31.8	±	6.13
P	631	IFPI 2355	Ethiopia	*sativum*	*arvense*	66.5	±	2.07	79.2	±	11.04	35.1	±	16.64

P	665	IFPI 3280	Syria	*sativum*	*syriacum*	63.1	±	14.88	83.9	±	15.64	35.7	±	16.35
P	657	IFPI 3252	Syria	*fulvum*		76.0	±	9.84	105.4	±	12.23	36.4	±	18.15
P	1566	PI 390784	Peru	*sativum*	*sativum*	73.8	±	1.76	56.5	±	3.62	36.7	±	27.91
P	649	IFPI 2441	Denmark	*abyssinicum*		79.1	±	6.35	70.2	±	7.79	38.2	±	11.33
P	37	PI 505080	Cyprus	*sativum*	*sativum*	81.2	±	5.59	74.4	±	6.71	38.3	±	14.78
P	659	IFPI 3257	Syria	*fulvum*		10.0	±	0.58	65.4	±	2.98	40.2	±	20.18
P	626	IFPI 2350	Ethiopia	*sativum*	*arvense*	72.9	±	3.67	87.5	±	7.20	40.4	±	29.98
P	660	IFPI 3260	Syria	*fulvum*		66.0	±	8.34		m		41.3	±	22.26
P	640	IFPI 2364	Ethiopia	*sativum*	*arvense*	75.1	±	6.29		m		41.6	±	11.43
P	647	IFPI 2371	Ethiopia	*sativum*	*arvense*	78.5	±	4.17	73.7	±	1.20	42.2	±	27.01
P	22	PI 344010	Greece	*sativum*	*elatius*	77.1	±	5.68	78.6	±	3.60	42.2	±	26.25
P	656	IFPI 3250	Syria	*fulvum*		73.9	±	4.10	91.7	±	4.22	45.1	±	15.06
P	642	IFPI 2366	Ethiopia	*sativum*	*arvense*	76.6	±	7.68	86.3	±	8.29	46.4	±	19.72
P	38	PI 505092	Cyprus	*sativum*	*sativum*	70.1	±	0.33	79.1	±	11.04	55.0	±	20.50
P	316	CGN 10193	Unknown	*sativum*	*arvense*	77.7	±	7.79	82.8	±	3.94	56.7	±	20.79
P	23	PI344011	Greece	*sativum*	*elatius*	56.8	±	12.15	87.5	±	7.26	59.4	±	37.90
P	39	PI 505111	Syria	*sativum*	*sativum*	72.9	±	5.84	57.7	±	14.45	59.5	±	28.41
P	315	CGN 10206	Unknown	*sativum*	*elatius*	78.4	±	5.52	74.4	±	6.72	67.4	±	34.21
P	16	PI 273209	Russia	*sativum*	*elatius*	76.6	±	9.78	95.9	±	4.18	69.5	±	14.10
P	1210	JI 1210	France	*sativum*	*sativum*	61.6	±	12.18	91.7	±	4.20	70.8	±	23.59
P	42	PI 268480	Afganistan	*sativum*	*arvense*	73.4	±	6.04	70.2	±	7.74	72.7	±	12.67
P	30	PI 242027	Denmark	*sativum*	*sativum*	73.0	±	8.23	73.2	±	8.76	72.9	±	35.15
P	31	PI 269762	UK	*sativum*	*sativum*	81.1	±	9.25	61.2	±	6.21	73.1	±	29.86
P	2302	BGE 025271	Spain	*sativum*	*sativum*	60.4	±	11.03	67.8	±	7.90	79.8	±	7.87

P	25	PI 344013	Greece	*sativum*	*elatius*	69.7	±	5.50	91.8	±	4.17	81.5	±	36.36
P	54	JI 804	Unknown	*sativum*	*arvense*	70.9	±	17.08	70.2	±	7.80	83.2	±	25.97
P	614	IFPI 3365	Turkey	*sativum*	*elatius*	76.2	±	8.43	87.0	±	0.60	84.1	±	21.25
P	36	PI 343988	Turkey	*sativum*	*sativum*	59.6	±	14.87	65.4	±	2.98	86.0	±	37.35
P	52	JI 254	Ethiopia	*sativum*	*elatius*	77.2	±	12.36	91.2	±	4.50	89.8	±	25.30
P	633	IFPI 2357	Ethiopia	*sativum*	*arvense*	72.5	±	20.67	65.4	±	7.81	90.2	±	30.63
P	634	IFPI 2358	Ethiopia	*sativum*	*arvense*	75.8	±	9.37	95.9	±	4.22	92.1	±	26.52
P	624	IFPI 2348	Ethiopia	*sativum*	*arvense*	69.3	±	15.08	74.3	±	6.72	94.7	±	13.03
P	34	PI 343985	Turkey	*sativum*	*sativum*	80.2	±	6.04		m		97.5	±	26.86
P	1747	PI 471349	India	*sativum*	*sativum*	79.2	±	4.67	104.8	±	4.78	98.3	±	17.61
Messire			France	*sativum*	*cv.*	100.0 (28.3)	±	0.01 (1.41)	100.0 (127.6)	±	0.0 (5.56)	100.0 (2.6)	±	0.00 (0.66)
P	632	IFPI 23656	Ethiopia	*sativum*	*arvense*	73.8	±	8.14	82.7	±	3.93	100.4	±	52.10
P	33	PI 505062	Greece	*sativum*	*sativum*	62.0	±	8.31	33.9	±	14.61	105.8	±	61.51
P	638	IFPI 2362	Ethiopia	*sativum*	*arvense*	81.7	±	7.82	73.7	±	1.20	108.9	±	10.17
P	62	JI 1429	Israel	*humile*		72.3	±	9.69		m		118.0	±	81.78
P	628	IFPI 2352	Ethiopia	*sativum*	*arvense*	68.5	±	7.40	75.0	±	7.21	119.5	±	56.00
P	68	JI 2201	Russia	*sativum*	*elatius*	66.1	±	11.94	69.7	±	10.88	126.0	±	62.22
P	7	PI 358609	Ethiopia	*abyssinicum*		70.7	±	12.37	58.9	±	22.61	128.3	±	19.60
P	17	PI 344003	Turkey	*sativum*		85.4	±	13.92	87.0	±	0.57	147.5	±	68.78
P	641	IFPI 23656	Ethiopia	*sativum*	*arvense*	78.1	±	5.94	83.4	±	8.31	147.5	±	76.59
P	646	IFPI 2370	Ethiopia	*sativum*	*arvense*	72.1	±	13.15	77.9	±	11.34	157.0	±	76.15
P	637	IFPI 2361	Ethiopia	*sativum*	*arvense*	74.6	±	7.51	91.0	±	4.51	159.7	±	61.71
P	650	IFPI 2495	UK	*abyssinicum*		76.6	±	8.95	82.7	±	3.93	161.9	±	73.11
P	629	IFPI 2353	Ethiopia	*sativum*	*arvense*	63.0	±	12.57	87.6	±	7.27	162.1	±	69.84
P	630	IFPI 2354	Ethiopia	*sativum*	*arvense*	87.4	±	7.45	74.4	±	6.76	188.9	±	63.79

P	14	PI 120617	Turkey	*sativum*	*elatius*	69.0	±	12.20	82.8	±	3.91	206.9	±	124.51
					Season Mean:	72.3 (20.3)			77.4 (98.4)			69.89 (0.30)		
					SE Mean:	1.18 (1.60)						4.89 (0.10)		
					d.f.:	1						1		

Actual values of RAUAIPC and RAUPDPC in brackets. SE, standard error of the differences between means; d.f., degrees of freedom associated to season mean

Table 5. Additional information of the four pea accessions evaluated under semi and controlled conditions.

Accession	Flower colour	Growth habit	Leaf type	Size	Flowering time	Detailed information
P29	Pigmented (2 distinct violet tones)	upright		med	med	https://npgsweb.ars-grin.gov/gringlobal/accessiondetail.aspx?id=1400063
P40	Pigmented (2 distinct violet tones)	upright		lar	lat	https://npgsweb.ars-grin.gov/gringlobal/accessiondetail.aspx?id=1400048
P665	Pigmented (violet)	creeping		sma	lat	https://www.icarda.org/tools/figs (web in progress)
cv. Messire	White	upright		med	med	Breeder: SERASEM S.A: http://www.samarisa.com/productos-agricultura.php?m=3&idc=20

lat: lately; med: medium; sma: small

CHAPTER 5

Antifeedant activity of fungal and plant metabolites against pea aphid (*Acyrthosiphon pisum*) as potential biocontrol strategy

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TITLE:

Antifeedant activity of fungal and plant metabolites against pea aphid (*Acyrthosiphon pisum*) as potential biocontrol strategy

ABSTRACT:

Aphids are noxious insect pests of major crops including cereals and legumes. In particular, pea aphids (*Acyrthosiphon pisum* Harris; Homoptera: Aphididea) is the cause of significant yield and quality loses in pea. Crop protection is largely based on chemical pesticides. The environmental pollution and the risks for human and animal health due to the massive use of chemicals have prompted a renewed interest in the discovery of natural products as alternatives to synthetic insecticides. In this study eight long chain alcohols and eight fungal and plant metabolites belonging to different classes of natural compounds were tested in dual choice bioassays to evaluate their feeding deterrence and mortality effect on pea aphids.

High feeding deterrence was produced by some of the compounds, particularly 1-hexadecanol, gliotoxin, cyclopaldic acid and seiridin. On the contrary, aphid mortality was low although significant for 1-heptadecanol, cyclochalasin A, 1-nonadecanol and gliotoxin. Some of the compounds damaged the plant but this phytotoxicity was low or imperceptible for cytochalasin A, seiridin and 1-nonadecanol.

The results obtained showed the potential of seiridin to be used in alternative to synthetic insecticides for the control of pea aphids as it deterred aphid feeding and was no toxic to the plant.

KEYWORDS: Pea aphids; long chain alcohols; fungal and plant metabolites; biocontrol

1 INTRODUCTION

Pea aphid (*Acyrthosiphon pisum* Harris; Homoptera: Aphididae), from now on abbreviated as PA, is a sucking phloem insect pest that can cause significant yield and quality loses in pea (*Pisum sativum* L.) crop. It has a broad host range, infecting also other legume species (Caillaud et al., 2002, Edwards and Singh, 2006a) such as faba bean, lupin (Schwartzberg et al., 2011b), alfalfa (Gao et al., 2008b) or lentil (Andarge and Westhuizen, 2004). Infestation of the host plants by aphids causes chlorotic damages, curly and wilting leaves, nutrient deficiencies and plant stunting.(Goggin, 2007) Furthermore, by nailing their stylus, cause an indirect and serious damage acting as viruses' vector.

The widespread and systematic use of chemicals and the use of biocontrol strategies does not provide the required control due to the complex PA life cycle (Blackman and Eastop, 2014) and their easy environmental adaptation (Tares et al., 2013, Srinivasan et al., 2014) and risk of development of resistance to pesticides (Vanlerberghe-Massutti and Guillemaud, 2007). Nowadays the overuse of chemicals as insecticides is being questioned, regarding the massive environmental damage, their human and animal health risks and application costs.(Coats, 1994, Schrader et al., 2010)

In front this scenario, seeking for alternatives to combat this pest is prompted. Recent studies demonstrate the capacity of natural compounds to combat several plant diseases and pests showing low or absent toxicity towards non-target organisms, biodegradability, and in many cases, economical production in comparison to those compounds produced by complete chemical synthesis.(Schrader et al., 2010, Barilli et al., 2017, Andolfi et al., 2013, Prota et al., 2014) Moreover the effect of natural substances over the development, survival and/or reproduction of PA has been reported, (Sauvion et al., 2004, Golawska et al., 2006, Sadeghi et al., 2009) which reinforce the potential of natural compounds as effective and non-harmful way to generate new biopesticides. A number of compounds have been described as antifeedant or deterrent, wthat means turn treated plants unattractive and unpalatable for aphids, or aphicides which cause pest mortality. (Golawska et al., 2008, Evidente et al., 2008, Evidente et al., 2009a, De Geyter et al., 2012, Golowska and Lukasik, 2012)

The objective of this study was to carry out a first evaluation of the feeding repellence and toxicity to PA of several long chain alcohols (LCOH) and metabolites (derived from different organisms) chosen on the basis of their biological activity.in order to select the most

interesting compounds for further analysis, with the long term goal of developing environmentally friendly PA management strategies.

2 MATERIAL & METHODS

2.1 Fungal and plant metabolites

Long chain alcohols (Table 1) and in particular 1-tetradecenol, 1-pentadecanol, 1-heptadecanol, 1-nonadecanol and 1-eiscosanol were purchased from Sigma- Aldrich (S. Louis, USA), while 1-hexadecanol, 1-octadeacanol and 1-nonadecanol from Loradan Fine Chemical (Sweden) while *trans*-9-octadecen-1-ol was obtained from reduction of methyl elaidate (purchased from Loradan Fine Chemical, (Sweden) by reduction as previously reported (Ganassi et al., 2016a). The fungal metabolites, namely, cyclopaldic acid, cytochalasins A and B, gliotoxin, 6-hydroxymellein, papyracillic acid and seiridin (Figure. 1 and Table 1) were purified from the culture filtrates of *Seiridium cupressi* (Graniti et al., 1992), *Pyrenophora semeniperda* (Evidente et al., 2002), *Neosartorya pseudofischeri* (Masi et al., 2013), *Phoma chenopodiicola* (Cimmino et al., 2013b), *Ascochyta agropyrina* var. *nana* (Evidente et al., 2009b) and *Seiridium cardinale* (Evidente et al., 1986) respectively. While the only plant metabolite tested, inuloxin A was obtained from the organic extract of *Inula viscosa* (Andolfi et al., 2013). The purity of all the metabolites was >98%, as checked by proton nuclear magnetic resonance spectroscopy (^{1}HNMR) and liquid chromatography–ESI mass spectrometry (LC-MS). The tested compounds were chosen, as mentioned before, for their biological activity. a) the long-chain alcohols that had previously shown phagodeterrent activity against the birdcherry-oat aphid *Rhopalosiphum padi*, a major pest of cereal crops (Ganassi et al., 2016a); b) the fungal and plant metabolites belong to different classes of natural compounds and show different biological activity. In particular: i) papyracillic acid, seiridin, cyclopaldic acid are fungal metabolites with not only showed antifungal activity (Graniti et al., 1992, Sparapano and Evidente, 1995, Evidente et al., 2009b, Barilli et al., 2016, 2017) but also biting deterrents activity against *Aedes aegypti* (Diptera: Culicidae)(Cimmino et al., 2013a, 2015a); ii) cytochalasins A and B showed a broad range of biological activities (Scherlach et al., 2010) including the recent reported significant biting deterrent activity against *Ae. aegypti* (Masi et al., 2017); iii) gliotoxin showed a broad range of biological activities (Borthwick, 2012) including strong biting deterrents activity against *Ae. aegypti*; (Masi et al., 2017) iv) 6-hydroxymellein showed nematocidal and antimicrobial activity (Stadler et al. 1995) but also biting deterrents activity against *Ae. Aegypti*

(Masi et al., 2017); v) inuloxin A is a plant metabolite showing antileishnanial activity against *Leishmania danovani* (Avolio et al., 2014).

Table 1. Long chain alcohol (LCOH) and fungal and plant metabolites used in the dual choice bioassay, with relative molecular weight and source.

Type	Compound		Molecular Weight	Source
Long chain Alcohols (LCOH)	**1**	1-Tetradecanol (TTD)	214	Sigma-Aldrich (S. Louis, MO, Usa)
	2	1-Pentadecanol (PTD)	228	"
	3	1-Hexadecanol (HXD)	242	Loradon Fine Chemical (Sweden)
	4	1-Heptadecanol (HPD)	256	Sigma-Aldrich (S. Louis, MO, USA)
	5	1-Octadecanol (OTD)	270	Loradon Fine Chemical (Sweden)
	6	*trans*-9-Octadecen-1-ol (OTD-trans)	268	Ganassi et al. 2016
	7	1-Nonadecanol (NND)	284	Loradon Fine Chemical (Sweden)
	8	1-Eicosanol (ECD)	298	Sigma-Aldrich (S. Luis, MO, USA)
Fungal and plant metabolites	**9**	Cytochalasin A		*Pyrenophora semeniperda*
	10	6-Hydroxymellein	194	*Phoma chenopodiicola*
	11	Cyclopaldic acid	238	*Seiridium cupressi*
	12	Seiridin	212	*Seridium cardinale*
	13	Inoluxin A	248	*Inula viscosa*
	14	Papyracillic acid	242	*Ascochyta agrpyrina* var. *nana*
	15	Cytochalasin B	479	*Pyrenophora semeniperda*
	16	Gliotoxin	326	*Neosartoria pseudofisheri*

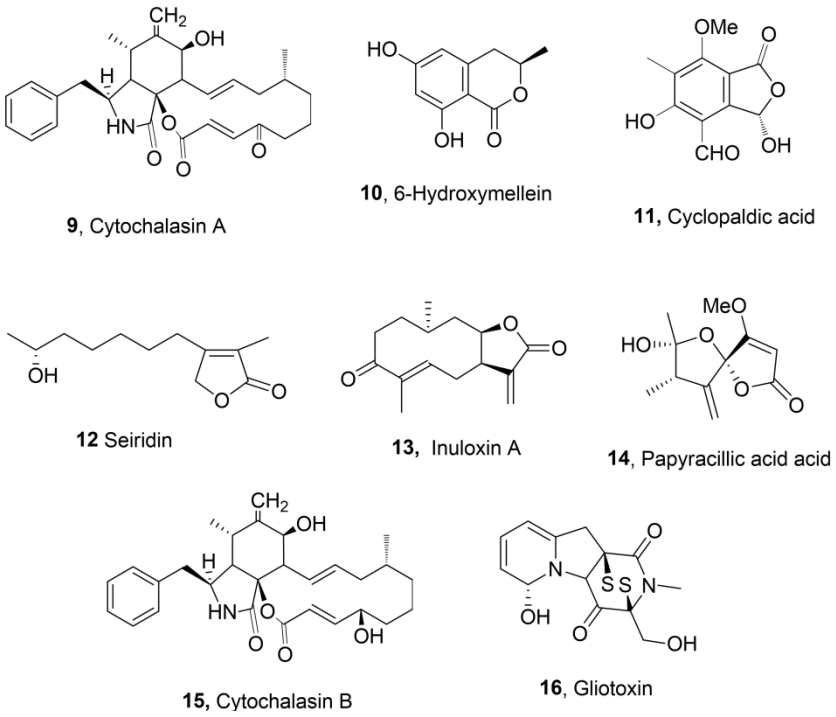

Figure 1. Structure of fungal and plant metabolites assayed.

2.2 Pea aphids and plant conditions

The pea aphid clone used in all the experiments derived from a single-aphid isolated in a pea experimental field from Córdoba, Spain. Aphids were maintained on faba bean (*Vicia faba* cv. Brocal) under chamber conditions (20 ± 2ºC; 65% RH and L12: D12 photoperiod). Bean is used for rear, because being a suitable host for pea aphid,(Franz et al., 1998) is less affected than pea to possible photoperiod and temperature alterations that can arise in the breeding chambers. Moreover its broad leaves allow to realize identical segments that could be completely flat over the agar of Petri dish, thus avoiding lumps and possible displacements of PA under the inoculated leaf surface. Nymphs of third and fourth instar were used for all bioassays. They were selected the day before and kept in darkness at 4ºC before use.(Aznar-Fernández and Rubiales, 2018 b)

Faba bean cv. Brocal seedlings used for the assays were grown under chamber conditions (20 ± 2 ºC; 65% RH and L14: D10 photoperiod) in plastic pots (10x10cm, 2 seeds) with a mixture of sand/perlite, 1:1 vol/vol.

2.3 Dual Choice assays

Pea aphids were subjected to a dual choice bioassay being offered for feeding on the same time two different options: leaves treated with a test compounds (option A) and leaves treated with a control solution (option B). In this study 16 different compounds long chain alcohols, derived from commercial sources, and fungal and plant metabolites (Table 1), were tested at two concentrations (0.5 and 1 mM), with 15 replications for treatment. Each replication consisted on 4 leaf segments (2cm diameter) of faba bean cv. Brocal placed adaxial side down equidistantly inside a Petri dish with a base of 5% water agar. Two leaf segments were painted with a paint brush with the test compound (option A) and the other two with the control solution (option B). To ensure that plant phenology did not influence on the PA selection, leaf segments always were taken from the fifth and sixth leaves of 3-week-old beans. Thereafter Petri dishes were placed under chamber conditions (20-22 ºC, 60% relative humidity and 12L:12D photoperiod) with a light intensity of 150 µmolm−2 s−1 photon flux density supplied by high-output white fluorescent tubes. Once Petri dishes were placed in the chamber, 15 apterous nymphs were transferred in the middle of each. To avoid light influence on aphids' mobility, dishes were covered by white paper. Being a dual choice assay with control solution present in both options (A and B) there is no need to check if control solution has an effect on PA feeding preference, since any notable effect will be due the metabolite used. However, 10 Petri dishes with 4 leaf segments arranged as above, 2 leaf segments painted with control solution and 2 leaf segments painted with distilled water were infested with 10 PA under same chamber conditions.

2.3.1. Test solutions

Test solutions were produced by diluting the compounds in MeOH 5% (v/v) and distilled water. To improve leaf wettability 0.02% of Tween-20 was added. Control solutions were prepared in the same way but no metabolites were added. After the inoculation, leaf segments were drain of the excess of solutions under laminar chamber flow (Figure 2a).

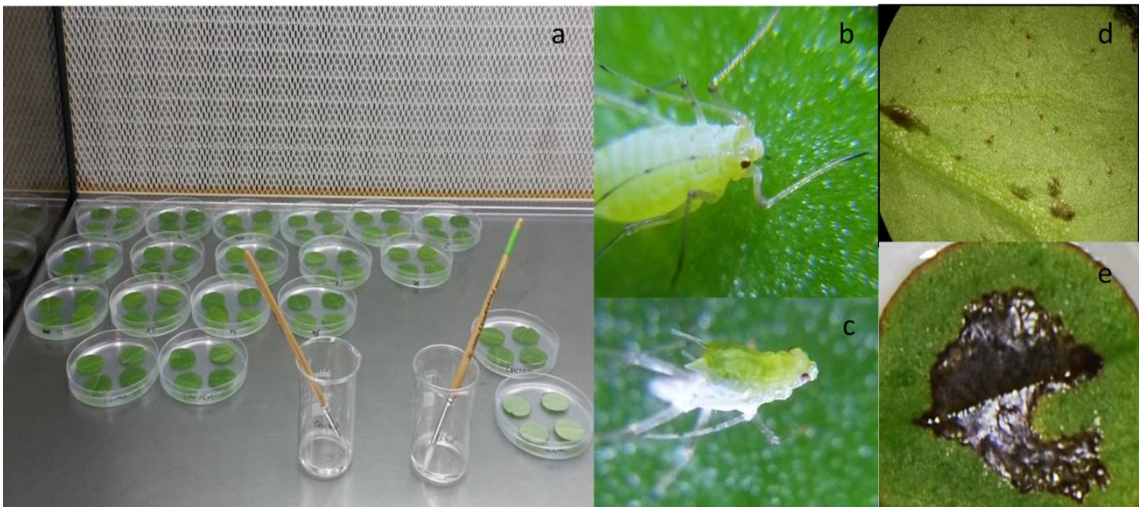

Figure 2. Explanatory images from the general procedure in dual choice assays performed. a) Assay procedure in flux chamber; b) Pea aphid feeding on a control fava bean circle; c) Aphid moulting during the assay; d) Control circles, abaxial part affected by PTD at 48 h after inoculation (visual scale = 2); e) Control cicles, adaxial part affected by gliotoxin at 96 hours after inoculation (visual scale =4). Pictures b, c, d and e were taken with a magnifying glass of 60X connected to a mobile phone (Samsung).

2.3.2. Aphid feeding and mortality assessments

Numbers of aphids feeding on each leaf segment were monitored at 0.5, 1, 2, 4 and 24 h after infestation (hai), assessing number of aphids feeding on control (FC) and treatment (FT) leaf segments. Care was taken to consider only sucking aphids (Figure 2b, where the stylet could be observed), excluding moulting aphids (in Figure 2c could be appreciated that moulting PA were not in contact with the leaf). Aphids showing discoloration, upside down, and that did not move when touched with a soft painting brush were considered dead. Thereafter, for each evaluation time the percentage of PA feeding over each treatment (FC and FT) was calculated and transformed by a logarithm formula. In addition Feeding Repellent Index (FRI%) was calculated to express the repellence ability of each metabolite (Kostić et al., 2013), using the following formula:

$$FRI\ \% = \frac{FC - FT}{FC + FT}\ X\ 100$$

The FRI% assumes positive values when the tested compound is a feeding repellent and negative value when it is a phagostimulant. Square root formula was used to transform FRI% data in order to follow a normal distribution.

Aphid mortality was assessed by counting the number of dead aphids on each evaluation time, so that it was possible to analyze the influence of time, compound and concentration. Natural death of aphids was not considered because of the reduced time of the assay as well as the PA selection done, by choosing the more vivacious.(Abbot, 1925) Data were referred to percentages of total aphids dead per repetition. In order to follow normal distribution data was transformed by [-1/log(%mortality)] formula.

2.3.3. Phytotoxicity assessment

Toxicity of the compounds was tested on non-aphid infested faba bean leaves, covering possible leaf damage in both surface parts. For this, 2 Petri dishes were painted with the compounds, one Petri dish containing 4 leaf segments in abaxial leaf side (Figure 2d) and the other containing 4 leaf segments in adaxial leaf side (Figure 2e). At the same time, with the same leaf disposition as described before, 2 Petri dishes were painted with control solution and 2 more with distilled water. No aphid infestation was done. Leaf damage (LD) were assessed at 24, 48 and 96 h after treatment (hat), using a visual scale (Prota et al., 2014): 0 = no damage to leaf surface; 1 = most of leaf surface normal, some sight pitting (extremely localized corrosion that leads to the creation of small holes); 2 = slight pitting over whole leaf surface or dry patches on leaf; 3 = slight pitting over whole leaf surface with some dry patches; 4 = pitting of leaf surface, large areas dry and papery; 5 = leaf shriveled. Data of LD was analysed by an area under phytotoxic progress curve (AUPPC), in order to integrate in the evaluation the three data collection,(Jeger and Viljanen-Rollinson, 2001) with the following formula:

$$AUPPC = \sum_{i=1}^{n-1} \frac{Y_{i}+Y_{i+1}}{2} \times (t_{i+1} - t_{i})$$

Where *Y* is the LD at assessment time point, *i* is the number of days after the first observation on assessment date and *n* is the number of successive observations. Thereafter, data was standardized (sAUPPC) by dividing the areas by total days of evaluation, multiplied by 100 to facilitate interpretation and transformed by square root to follow a normal distribution.

2.4. Statistical analysis

Statistical analyses were developed using Statistix 10 ® (Analytical Software, Tallahassee, USA). Mean differences for aphid % of mortality along time were calculated by HSD-All-Pairwise comparison test ($\alpha \leq 0.05$).

3. RESULTS

ANOVA for % of feeding aphids revealed a significant effect of time (T) followed by compound (CO) and concentration (C), with lower influence of CO x C and C x T interactions (Table 2). Due to the high influence of T on the amount of feeding aphids, we proceeded to calculate the greatest evaluation time for aphid feeding, being significantly higher 24 hai (67.5%). Therefore data of FRI % and % of mortality by 24hai were analyzed. Dual choice assay between segments of leaves painted with control solution *vs* distilled water, showed no significant differences for aphid feeding preference ($P>0.05$).

Table 2. Analyses of variance of % *Acyrtosiphon pisum* feeding on faba bean leaf segments treated with 16 different compounds (CO), at 5 different times (T) under 2 different concentrations (C) 0.5 and 1 mM. (DF: degrees of freedom; SS: sum of square; MS: mean square).

Source	DF	SS	MS
Compound (CO)	15	80634	53756**
Concentration (C)	1	1450	14495**
Time (T)	4	281190	702976**
CO x C	15	9108	0,6**
CO x T	60	24657	0,4**
C x T	4	0,7	0,2
CO x C x T	60	12121	0,2
Error	2130	490931	0,2
Total	2289		

Concerning to FRI %, ANOVA with CO and C as fixed factors (Table 3), revealed significant differences for CO ($P<0.05$) (Figure 3). The most repellent compound was number **3,** a LCOH, (FRI% 67.6 %) at 1mM, followed by metabolites **16** (54.0 %), **11** (48.46 %) and **12** (47.6 %), the last two at 0.5 mM. On the contrary compounds **4** and **6**, both LCOH, were

phagostimulants (-71.4 % and -50.43 %, respectively) at the higher concentration used (1 mM) with little effect at 0.5 Mm.

Table 3. Analyses of variance of feeding repellent index (FRI%) and % mortality of *Acyrtosiphon pisum* and standarized área under phytotoxic progress curve (sAUPPC) over 16 different compounds (CO) and 2 different concentrations (C) 0.5 and 1 mM (DF: degrees of freedom; SS: sum of square; MS: mean square).

	Source	DF	SS	MS
%FRI	Compound (CO)	15	36,4	0,2**
	Concentration (C)	1	0,04	0,0
	CO x C	15	13,1	0,1
	Error	282	244,8	0,1
	Total	313		
% Mortality	Compound (CO)	15	61955	0,4**
	Concentration (C)	1	0,1	0,1
	CO x C	15	1,0	0,1**
	Error	337	103668	0,0
	Total	368		

** Statistically significant (P < 0.05)

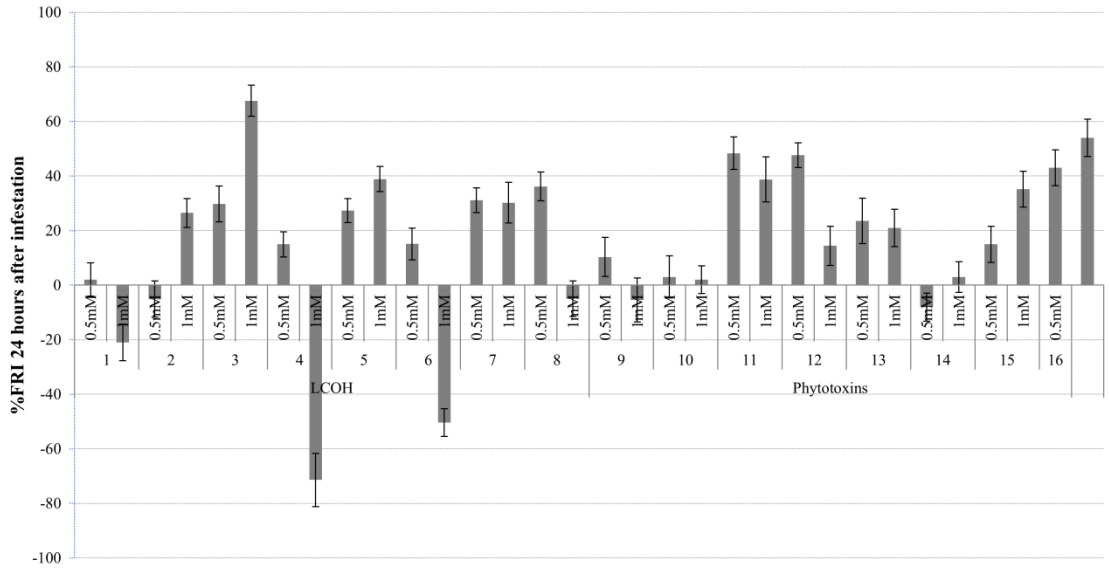

Figure 3. Feeding repellent index percentage (FRI%) of pea aphid for 16 metabolites, tested in a petri dish assay, 24 hours after inoculation (hai). The compounds were tested under 0.5 mM and 1 mM concentrations. The experiment was repeated 15 times, and the standard deviation was calculated.

Regarding aphid mortality, it increased with time for all treatments being maximal at 24 hai (Figure 4). ANOVA for mortality 24 hai, with CO and C as fixed factors (Table 3), revealed significant differences for CO (P=0.001) with lower influence for CO x C interaction and no significant C influence. In spite of significant differences, values were in general low, ranging from a maximum of 4.2% for metabolite **4** to 0.15% for compound **12,** both at 1 mM.

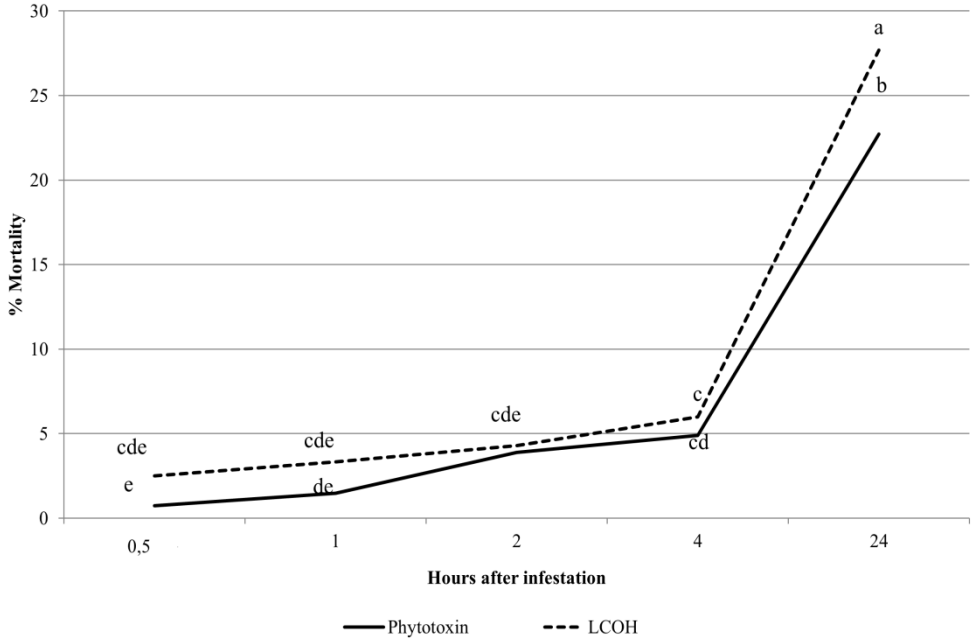

Figure 4. Percentage of pea aphid mortality along time, according to the type of compound tested. Different letters over each time point showed significant differences HSD test (alpha=0.05).

Data from FRI% and % mortality for compound and concentration is shown in Table 4. There was no correlation between FRI% and mortality for each metabolite (r=0.17; P=0.08).

Concerning to the phytotoxic effect of compounds, no damage was observed in the controls showing nine of the compounds imperceptible leaf damage. ANOVA, carried out with the compounds that showed perceptible LD, showed significant differences for CO (P=0.001), but not for C neither leaf surface part (Lsp) (P>0.05). As Lsp showed no significant interactions with metabolites, values shown in Table 5 are the global mean of the segments inoculated with the same metabolite for each concentration. The compounds with higher phytotoxity were: Gliotoxin (nAUPPC 261 and 223 for 0.5 and 1 mM, respectively), Cyclopaldic acid (109

and 79, respectively) and NND (93 and 72, respectively), showing Papyracillic acid LD only under 1mM (29).

Table 4. Feeding deterrence index percentage (FRI%) and mortality percentage with standard error calculated (SE), of 16 different compounds at two concentrations (C) for *Acyrtosiphon pisum* 24 hai. Assays performed on faba bean (cv. Brocal) under growth chamber conditions. (Figure 2)

	Compound	C (mM)	FRI% ±SE	%Mortality ±SE
LCOH	**1**	0.5	2.0±12.13	1.3±0.34
		1	21.0±13.47	1.0±0.42
	2	0.5	-5.1±13.33	1.7±0.34
		1	26.5±10.57	2.2±0.32
	3	0.5	29.8±13.12	3.5±0.09
		1	67.6±11.43	3.4±0.12
	4	0.5	14.9±9.26	3.5±0.40
		1	71.4±19.41	4.3±0.14
	5	0.5	27.3±8.87	2.2±0.26
		1	38.9±9.22	2.9±0.13
	6	0.5	15.1±11.62	3.2±0.17
		1	50.4±10.17	3.1±0.26
	7	0.5	31.1±9.06	3.5±0.07
		1	30.2±14.8	3.6±0.09
	8	0.5	36.2±10.56	3.1±0.13
		1	-5.0±13.01	3.0±0.16

	Compound	C (mM)	FRI%±SE	%Mortality ±SE
Fungal and plant metabolites	**9**	0.5	10.3±14.27	3.8±0.09
		1	-5.4±16.19	3.7±0.09
	10	0.5	3.0±15.44	1.8±0.46
		1	2.0±10.14	2.8±0.32
	11	0.5	48.4±11.83	1.8±0.53
		1	38.7±16.50	2.7±0.37
	12	0.5	47.6±9.00	2.2±0.24
		1	14.4±14.24	0.6±0.15
	13	0.5	23.5±16.54	2.2±0.39
		1	20.9±13.72	2.3±0.34
	14	0.5	-8.0±10.31	2.7±0.15
		1	3.0±11.2	1.0±0.13
	15	0.5	14.9±13.12	2.6±0.37
		1	35.2±13.12	3.2±0.26
	16	0.5	43.0±13.12	3.5±0.09
		1	54.0±13.64	3.4±0.09

Table 5. Phytotoxic effect over *faba bean* leaf segments of 16 compounds tested under 2 different concentrations (0.5 and 1 mM). Compounds are ordered by type.

		nAUDPC mean ± SE[a]	
Type	**Compound**	**0.5mM**	**1mM**
Phytotoxin	Gliotoxin	261.0 ± 13.2	223.4 ± 21.7
	Cyclopaldic Acid	109.4 ± 13.1	79.0 ± 11.5
	Cytochalasyn B	26.6 ± 5.5	18.6 ± 2.4
	Papyracillic	0.0 ± 0.0	29.2 ± 11.8
	Hydroxi-medellin-6	25.0 ± 0.3	25.4 ± 0.5
	Inoluxin	0.0 ± 0.0	0.0 ± 0.0
	Cytochalasin A	0.0 ± 0.0	0.0 ± 0.0
	Seridin	0.0 ± 0.0	0.0 ± 0.0
LCOH	HXD	9.3 ± 1.8	72.2 ± 8.7
	NND	65.5 ± 19.0	45.3 ± 14.3
	HPD	0.0 ± 0.0	0.0 ± 0.0
	OTD	0.0 ± 0.0	0.0 ± 0.0
	ECD	0.0 ± 0.0	0.0 ± 0.0
	TTD	0.0 ± 0.0	0.0 ± 0.0
	OTD-trans	0.0 ± 0.0	0.0 ± 0.0
	PTD	0.0 ± 0.0	0.0 ± 0.0

[a]Normalized area under phytotoxic progress curve and SE standard of the mean error

4. DISCUSSION

Pea aphid control is based on chemical insecticide worldwide. In a search of alternative natural products with potential applications in future management strategies we identified several compounds belonging to different classes of natural substances, causing significant feeding deterrence and/ or small levels of mortality. 1-Tetradecanol, 1-pentadecanol, 1-hexadecanol, 1-heptadecanol, 1-octadecanol, *trans*-9-octadecenol, 1-nonadecanol and 1-eicosanol (**1**-**8**, Table 1) are long chain alcohols, cytochalsins A and B (**9** and **15**, Figure 1 and Table 1) are [24]oxa-14 cytochalasans, 6-hydroxymellein (**10**, Figure 1 and Table 1) is a trisubstituted isocoumarin, cyclopaldic acid (**11**, Figure 1 and Table 1) is a pentasubstituted isobenzofuranone, seridin (**12**, Figure 1 and Table 1) is a 3,4-dialkylfuran-2-one, inuloxin A (**13**,

Figure 1 and Table 1) is a germacrane sesquiterpene, papyracillic acid (**14**, Figure 1 and Table 1), is a pentasubstituted 1,6-dioxaspiro[4.4]non-3-en-2-one and gliotoxin (16, Figure 1 and Table 1) is substituted diketopiprazine.

Cyclopaldic acid and seiridin at 0.5 mM and 1-hexadecanol and gliotoxin at 1 mM, showed high aphid feeding deterrence. Metabolites studied caused in general little aphid mortality being significantly higher for 1-hexadecanol, cytochalasin A both at 0.5 mM and 1-heptadecanol and gliotoxin at 1 mM. Some of these caused significant phytotoxicity on faba bean leaves, but cytochalasin A, seridin, 1-nonadecanol and 1-hexadecanol were not phytotoxic, reinforcing their value as future biopesticides.

Feeding deterrence and mortality increased markedly from 4 hai, being maximal at 24 hai, indicating that activity of compounds over PA should be evaluated from 4 hai. In addition, for both traits the concentration of the compounds used was the least influential, which reinforce the activity of the compounds used.

Results elucidated that LCOH showed drastically changing values for FRI% than fungal and plant metabolites, this differences were pronounced at 1mM concentration. This could be explained because LCOH have been described as signal molecules in several insects groups,(Ganassi et al., 2016b) and under higher concentrations power their activity, such as in 1-hexadecanol (compound num. **3**) by showing the maximum level of feeding deterrence activity and 1-heptadecanol (compound num. **4**), with the highest feeding attraction at 1 mM. Same conjecture could explain the greater mortality 24 hai in the LCOH metabolites. Among the most outstanding LCOH was 1-hexadecanol, which showed at 1 mM a great combination of high FRI % and % of mortality, but performed a moderate sAUPPC value on leaf damage (LD), which discourages its use for biocontrol under these concentrations in faba. On the other hand 1-heptadecanol performed at 1 mM the highest feeding attraction (or lower FRI %) and % of mortality; which could be translated as a deadly trap for aphids and negligent LD. However as PA mortality was too low, 1-heptadecanol is not suitable for PA control. Another metabolite with high % of mortality was 1-nonadecanol (compound num. **7**), but showed low FRI % and intermediate LD, which made it less conducive.

Four fungal metabolites were identified having deterrence activity. These were gliotoxin (**16**), cytochalasin A (**9**), cyclopaldic acid (**11**) and seridin (**12**). Gliotoxin is a mycotoxin with several biological activities such as antiviral, immunomodulatory,(Tuch et al., 1988) antifungal (Coleman et al., 2011) and its ability to deter *Aedes aegypti* mosquito bite (Masi et al., 2017) Gliotoxin showed the highest feeding deterrence and mortality although was also the most

phytotoxic, limiting its value as bio pesticide. Cytochalasin A (*cytos* = cell, *chalasis* = relaxation) is a mold metabolite, which comes from a generalist minor plant pathogen. It has been described as a disruptor of the actin cytoskeleton,(Kuo and Lampen, 1975) inhibitor of spore germination and hyphal tip growth on some fungus(Stanley and Sweigardab, 1980) and inhibitor of sugar uptake and growth on some yeasts, as well as recently described as biting deterrent to *Aedes aegypti*.(Masi et al., 2017) Cytochalasin A showed little deterrence although was the one showing highest mortality in spite of little phytotoxicity. Still, as although significant, the levels of mortality were too low as for 1-heptadecanol to justify its use in PA control. In contrast, cyclopaldic acid and seridin showed high deterrence. Both of them have been described as deterrents against the mosquito *Aedes aegypti*.(Cimmino et al., 2013a), cyclopladic acid has been described as inhibitor of *U. pisi* and *P. triticina* growth (Barilli et al., 2017). Cyclopaldic acid was phytotoxic on faba bean, but seiridin was not. For this reason seiridin is a better option to conduct a biocontrol strategy. Seiridin originated from a micro-fungus that causes a lethal canker disease on cypress and other related conifers (Graniti, 1998) and it is known also for its low antibacterial activity.(Sparapano and Evidente, 1995)

Further bioassays are needed to clarify the effectiveness of this metabolites as bio pesticide under field conditions, as the dose of compound received in this assays is much higher than would be expected from a sprayed surface and to discern if they are specific, or could also damage mammals or other insects, in order to produce healthy commercial bio pesticides. It would be also desirable to carry out EPG studies (Golawska, 2007) to obtain more in-depth information. In addition obtain natural phytotoxic products is advantageous as having great variety of carbon skeleton and different functionalities are able to overcome the resistance that could be developed against synthetic pesticides.

In addition some fungal and plant metabolites are known as phytotoxins but it is important to take into account that many toxins are not selective, being able to cause different toxic effects both on host and non host plants (Evidente et al., 2011, Cimmino et al., 2015b) as seen in our results (Table 5). For this reason, the phytotoxicity was again evaluated on faba bean and interestingly, we found significant feeding deterrence in Seiridin, with not phytotoxicity for the crop. The identification of a natural compound potentially repellent with no phytotoxic effects is a breakthrough to control PA, for its potential application to aphid management in legume crops.

The adverse effects of synthetic pesticides prompted the search for natural compounds with antifeedant and/or mortal activity on pea aphid. Seiridin (**12**, Figure 1 and Table 1) highlighted with significant deterrence and imperceptible leaf damage.

These results showed the potential of Seridine to be used as alternative to synthetic insecticides for the control of crop pathogens of economic importance as PA. Moreover, in this study glyiotoxin, cyclopaldic acid and 1-hexadecanol compounds performed an interesting PA deterrence, which suggest them for future studies, in order to decrease or eliminate its leaf damage, and thus be able to take advantage of its powerful deterrent and mortal activity.

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General Conclusions

1. Resistance to *Bruchus pisorum* and *Acyrthosiphon pisum* is available in *Pisum* spp. germplasm as shown by screenings performed under field conditions. This includes both accessions of *P. sativum* ssp. *sativum* and of other subspecies and species that have already been crossed with pea cultivars, making this resistance readily accessible to pea breeding.

2. Resistance to *B. pisorum* is based on antixenosis and/or antibiosis mechanisms, resulting in reduced seed infestation and hampered larval development.

3. Level of reduction of seed infestation and larval development were not correlated, suggesting the inability of *B. pisorum* females to discern the appropriateness of seed during oviposition.

4. *B. pisorum* oviposition and larval development are heavily affected by environment and climatic factors. Canonical correspondence analysis showed that both decreased in rainy seasons, meanwhile accumulated radiation and photoperiod favoured seed infestation and decreased larval development.

5. Choice and non-choice assays with selected pea accessions from field assays elucidated the influence of pollen and pod genotype on *B. pisorum* oviposition.

6. Linkage mapping studies showed three QTL governing reduction of *B. pisorum* seed infestation and one for hampered larval development. Expressions of these QTL were influenced by the environment. Associated markers can assist in reducing efforts in plant breeding programs as well as increasing the knowledge of pea genome involved on defence.

7. Resistance in pea against *A. pisum* is based in a combination of antixenosis and antibiosis mechanisms. Accession P40 (*P.s*. ssp. *sativum*) highlighted by its antibiosis by diminishing drastically pea aphid development causing little plant damage. Meanwhile accession P665 (*P.s*. ssp. *syriacum*) showed antixenosis mechanism by repealing pea aphid presence.

8. Activity of fungal and plant metabolites against pea aphid identified seiridin (a toxin from *Seridium cardinale* fungus) as a potential metabolite to be used as an alternative to synthetic insecticide, with significant deterrence and imperceptible leaf damage. Three additional metabolites were also identified for its deterrence, but caused high leaf damage.

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